REVIEW OF LITERATURE

World wide problem of persistent pesticides

Pesticides have been used worldwide to control pests and pathogens for sustainable food productivity that is necessary to support the world population of approximately 7 billion people. Although the use 3of pesticide has a long history, the first documented use to control pests dates back to 2500 BC. However, in the past 50 years the use of chemical control has increased tremendously (Day et al., 1997). The earliest pesticides that were used included either inorganic or plants derived products. The modern era of chemical pest control started during World War II, when the much maligned DDT played a major role in the health and welfare of soldiers who used it to control body lice and mosquitoes which transmitted major illnesses. Further developments of pesticides followed. Owing to their low cost, ease of application and their effectiveness, pesticides have become the primary means of controlling pest. However, many pesticides are persistent organic pollutants (POPs). POPs are defined as chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment. As a consequence, increased use of these chemicals has caused considerable environmental pollution and human health problems (Kurzel and Certrulo, 1981; Hayes, 1986; Jit et al., 2011; Vijgen et al., 2011). Hence, contamination of soil, sediment, ground waters and surface waters by POPs is as an important environmental concern world-wide.

Pesticides

Humans are exposed to environmental pollutants such as pesticides either by use in agriculture, industrial production site, medicinal, etc. *Pesticides* are defined as "Any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit. Also used as substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport" (FAO; 2002).

Classification of Pesticides

Pesticides can be classified based on the target organism, chemical structure, and physical state. On the bases of chemical structure, pesticides are classified as inorganic pesticides (copper sulphate, ferrous sulphate etc.) and organic pesticides. Organic pesticides includes biological (botanicals), microbial (fungi that control certain weeds, subspecies and strains of *Bacillus thuringiensis* which kill specific insects etc.) pesticides, plant derived compounds (pyrethroids, rotenoids, nicotinoids etc), and synthetic pesticides

(Organophosphate Pesticides, Carbamate Pesticides, Organochlorine Insecticides and Pyrethroid Pesticides).

Organochlorine pesticides

Organochlorine insecticides were commonly used in the past, but many of these pesticides have been discontinued due to their health and environmental effects and persistence (e.g. DDT and chlordane, HCH, endosulfan, etc). Organochlorine insecticides are amongst the oldest, most toxic, and most environmentally harmful synthetic pesticides. First introduced in the 1940s and 1950s, these chemicals were used extensively in agriculture, forestry, and in home pest control until most were banned in the 1970s and 1980s. Organochlorine pesticides (OCPs) are classified into three groups: Hexachlorocyclohexane isomers (e.g., Lindane), cyclodienes (aldrin, dieldrin, endrin chlordane, heptachlor, and endosulfan) and DDT and its analogs (methoxychlor, dicofol, and chlorobenzylate). Many of these agents such as aldrin, lindane, endosulfan, etc., have been banned in various countries, but still used due to their low cost and effectiveness. Organochlorines like lindane also tend to be very persistent, breaking down exceedingly slowly once released into the environment. This property made them attractive insecticides, since often just single application would be effective for months. But this also makes them especially toxic to humans and the environment, as it can be incorporated into ecosystems, food chains and persists for years. These persistent pollutants tend to be poorly soluble in water but highly soluble in fat, properties that result in their storage in the fatty tissues of animals (a phenomenon known as

bioaccumulation), causing serious health problems. The present study is focused on the degradation of the organochlorine pesticide, lindane.

Lindane (γ–Hexachlorocyclohexane)

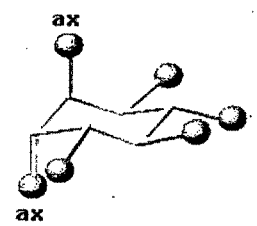
Lindane was named after the Belgian chemist Van Der Linden who discovered it in 1912 (Ulman, 1972). 1, 2, 3, 4, 5, 6-Hexachlorocyclohexane is referred as "HCH" by the WHO. The term 'Lindane' is used for the γ–isomer of Hexachlorocyclohexane (HCH), whereas 'technical HCH' refers to the mixure of the all isomers of HCH. Lindane or HCH has historically and widely but inappropriately referred to as "benzene hexachloride" also with the abbreviation as "BHC" by UN FAO and ISO.

History of the Lindane

Lindane and other isomers of hexachlorocyclohexane (HCH) were one of the most extensively used organochlorine pesticides produced mainly after the Second World War until the 1990s (Breivik et al. 1999). The application of Lindane and technical HCH during the last six decades has resulted in environmental contamination of global dimensions (Li 1999; Li et al. 2003; Li and MacDonald 2005; Vijgen, 2006, Vijgen et al., 2011). Lindane (γ–HCH) is synthesized by chlorination of benzene in the presence of ultraviolet light (IARC, 1973). This process results in technical grade HCH, which is a mixture of four major isomers: α-HCH (60-70%), β-HCH (5-12%), γ-HCH (10-15%) and δ-HCH (6-10%) (Kutz et al., 1991) (Table1). Besides these isomers, four other isomers produced during lindane synthesis, but they are present either in trace amount or are unstable. The isomers differ in the position of the chlorine atoms (Figure 1). Of all HCH

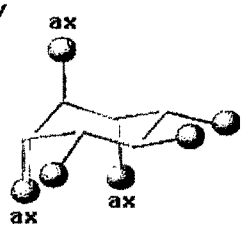
Figure 1: Structures of α-, β-, γ- and δ-HCH isomers. Spheres represent chlorine atoms. Isomers differ according to the number of axial (ax) positioned chlorine atoms (3 for γ-HCH, 2 for α-HCH, 1 for δ-HCH and 0 for β-HCH)

α



β

γ



δ

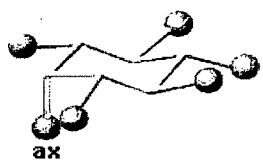


Table 1: Physical and chemical properties of Lindane and other HCH isomers (Kutz et al., 1991)

Name	γ-HCH	α-HCH	β-HCH	δ-HCH
Molecular weight	290.83	290.83	290.83	290.83
Colour	White[a]	Brownish to white[b]	No data	No data
Physical state	Crystalline solid[c]	Monoclinic prisms[d]	Crystalline solid[c]	Fine plates[e]
Melting point (°C)	112.5[e]	159-160[e]	314-315[e]	141-142[e]
Boiling point (°C)	323.4 at 101.3 kPa[e]	288 at 101.3 kPa[d]	60 at 67 Pa[e]	60 at 48 Pa[e]
Density (g $[cm^3]^{-1}$)	1.89 at 19°C[f]	1.87 at 20°C[e]	1.89 at 19°C[e]	No data
Odour	Slightly musty[g]	Phosgene-like[g]	No data	No data
Solubility				
in water	10 mg l^{-1}[h]	10 mg l^{-1}[h]	5 mg l^{-1}[h]	10 mg l^{-1}[h]
in ethanol	64 g kg^{-1}[h]	18 g kg^{-1}[h]	11 g kg^{-1}[h]	244 g kg^{-1}[h]
in ether	208 g kg^{-1}[h]	62 g kg^{-1}[h]	18 g kg^{-1}[h]	354 g kg^{-1}[h]
in benzene	289 g kg^{-1}[h]	No data	19 g kg^{-1}[h]	414 g kg^{-1}[h]
Log K_{ow}	3.72[i]	3.8[i]	3.78[i]	4.14[i]
Vapour pressure	5.6 10^{-3} Pa at 20°C[b]	6.0 10^{-3} Pa at 25°C[b]	4.8 10^{-5} Pa at 20°C[b]	4.7 10^{-3} Pa at 25°C[b]
Henry's law constant (atm m^3 mol^{-1})	1.4 10^{-5}[j]	1.06 10^{-5}[j]	7.43 10^{-7}[k]	2.1 10^{-7}[l]

[a](Osol, 1980) [b](HDSB, 2003) [c](IARC, 1979) [d](Weast, 1979) [e](Lide, 1991) [f](Weiss, 1986) [g](Merck, 2001) [h](Clayton and Clayton 1981) [i](Hansch et al., 1995) [j](USEPA) [k](Ripping, 1972)[l](Pankow et al., 1984)

isomers, only the γ-isomer has specific insecticidal properties. Due to this, pesticide producing companies from 1950s began to purify γ-HCH (99% purity from most suppliers), which was/is used under the name Lindane (Willet et al., 1998; Walker et al., 1999). Few countries adapted to this change much later than the 1950s. For example, in India technical HCH was used until the late 1990s and later the production and use of Lindane began. In China, the use of technical HCH was banned in 1983, and Lindane use began in 1990 (Li et al., 2001). Because of its suspected carcinogenic, persistent, bioaccumulative and endocrine disrupting properties (ATSDR 1998; ATSDR 2005; UNEP 2005a, b; WHO 1991), Lindane has become a heavily scrutinized substance and has been flagged for regulatory intervention in recent decades (Hauzenberger 2004). The use of Lindane has been banned in at least 52 countries and restricted by some of other countries including India. In 2009, an international ban on the use of α-HCH, β-HCH, and Lindane (industrial γ-HCH) in agriculture was implemented under the Stockholm Convention on Persistent Organic Pollutants (Vijgen et al., 2011). However, specific exemption allows for it to continue to be used in second-line treatments for the head lice and scabies.

Recent Lindane production

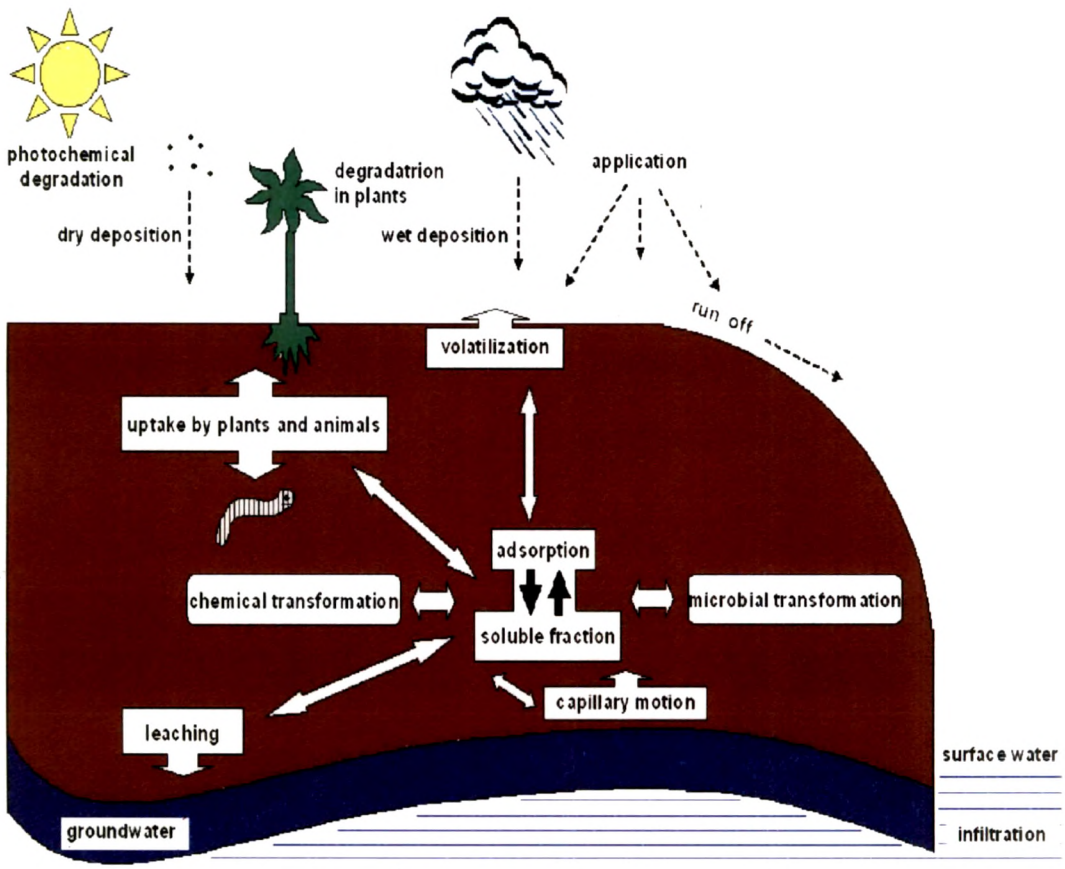
Globally the use of Lindane for agricultural, pharmaceutical and other applications from 1950 to 2000 is estimated to have been in the range of 600,000 tonnes (Vijgen et al., 2011). However, the global production of Lindane has decreased significantly in recent years. The production of technical HCH has been effectively ceased in India since 1997

through a national ban on technical HCH use. Recently, Lindane has been (Banned vide Gazette Notification No S.O. 637(E) Dated 25/03/2011) banned for Manufacture, Import or Formulation w.e.f. 25th March, 2011 and for its use w.e.f. 25th March, 2013 (http://www.cibrc.gov.in/list_pest_bann.htm) in India. Earlier Kanoria Chemicals Ltd (1,000 t/a capacity) and India Pesticide Ltd (300 t/a capacity) were the major producers of Lindane in India (IPEN 2005; Abhilash and Singh 2009a, b). The production and storage practises of Lindane producers in India were reported to be not adequate, leading to pollution around HCH producing and its associated waste storage sites (CAPE 2005; Abhilash and Singh 2009a, b; Jit et al. 2011). According to previous industrial production data, still 56,000 tonne stored/deposited HCH waste isomers are present in the country (Vijgen et al., 2011).

Lindane (and other HCH isomers) in the environment

As a result of the extensive use of technical HCH and Lindane over the past decades, Lindane and its waste isomers are widely distributed in various environmental compartments. The various transportation and transformation processes that are involved in the distribution of a pollutant in the environment are schematized in Figure 2. Once in the environment, pollutants are subject to various transportation and transformation processes (HCN, 1996). Although the HCH isomers only differ in the orientation of their chlorine atoms, relatively large differences exist in their physical-chemical properties (Table1). This implies different transport and persistence characteristics. However, this is not an exhaustive list, since in literature; data on concentrations and the persistence of HCHs in the various environmental compartments remain controversial. This is most

Figure 2: Various transportation and transformation processes involved in distribution of a pollutant in the environment. (HCN, 1996)


photochemical degradation
dry deposition
degradatrion in plants
wet deposition
application
run off
volatilization
uptake by plants and animals
adsorption
chemical transformation
microbial transformation
soluble fraction
capillary motion
leaching
groundwater
surface water
infiltration

likely due to the wide concentration ranges that can be found and the complex interactions that influence both biotic and abiotic removal.

HCH in the atmosphere

The major sources of HCH isomers in the atmosphere are fugitive dust particles from wind erosion of contaminated soil, volatilization from treated agricultural soil and from plant foliage sprayed with Lindane. In addition, HCHs may be emitted into the atmosphere during the manufacturing and reformulation process. Degradation by reaction with photolytically generated hydroxyl radicals, on the other hand, is an important process that removes Lindane and other HCHs from the atmosphere (Unsworth et al., 1999). HCHs can also be removed from the atmosphere by rain (wet deposition) and dry deposition (Atkins and Eggleton, 1971). HCHs can remain in the air for long periods of time and travel great distances depending on the environmental conditions. In the atmosphere, residence times for HCHs of 2 to 4 months have been reported (Brown, 1978). These extended residence times may cause a long range transportation of HCHs. For example, the volatilization of Lindane (or technical HCH) after its application in tropical or sub-tropical countries and subsequent transportation by air masses, is known to have resulted in the deposition of HCHs as far as on the Arctic (Takeoka et al., 1991). Mainly γ- and α-HCH are found in the atmosphere, due to the higher volatility compared to β- and δ-HCH (Table 1, Henry's law constants). Mean global concentrations of 580 pg m^{-3} γ-HCH and 1021 pg m^{-3} α-HCH in air have been reported by Walker et al. (1999) in his studies from 1980 to 1996. Peak concentrations as high as 10,000 pg m^{-3} were found over the Bay of Bengal and the Arabian Sea (Cotham and Bidleman, 1991). HCH

concentrations of 1.45 to 35.6 ng m^{-3} have been reported in the air from a tropical coastal environmental at Parangipettai southeast coast of India (Rajendran et al., 1999).

HCH in water

From Table 1, it is clear that HCHs are relatively soluble in water, at least compared to most other organochlorine compounds (e.g. DDT, aldrin, endrin, dieldrin, endosulfan have a solubility of <1 mg l^{-1}). Through leaching HCHs can reach the groundwater and contamination of surface water may occur as a result of surface run-off (as dissolved chemicals are absorbed to particles) and atmospheric depositions. The low solubility of β-HCH and the high sorption constant of δ-HCH (Table 1) cause the more frequent detection of the γ- and α-HCH in water bodies. Biodegradation of γ - and α-HCH in aquatic systems is considered the most dominant process in the removal mechanism of HCHs from water. The estimated degradation half-lives of these HCHs in rivers, lakes and groundwater are 3-30 days, 30-300 days, and >300 days, respectively (Zoetemann et al., 1980). In India, HCH residues have been reported in drinking water and soft drinks (Mathur et al., 2003a and 2003b). Further, HCH residues were also reported in the Cauvery River water, which is one of the major rivers of South India (Begum et al., 2009). Water samples have been collected from rural areas of Gujarat State, India, found to be contaminated with hexachlorocyclohexane (HCH) residues.

HCH in sediment

While in the water column, HCHs may be adsorbed to sediment or other suspended materials, which is evidenced by the relatively high K_{ow} values (Table 1). The adsorption

and desorption rate of HCHs to sediment is related to the physical characteristics of the sediment as well as to its organic carbon content. According to the USEPA's database of sediment samples collected throughout the United States between 1978 and 1987, γ-HCH was detected in 0.5% of about 600 sediment samples at a median concentration of ≤2.0μg kg-1 (Staples et al., 1985; Kuntz and Warry, 1983). The half-life of Lindane in sediment has been estimated at 90 days (Bintein and Devillers, 1996). Other HCH isomers have also been frequently detected in sediment samples (HazDat, 2005). Further HCH residues were also reported in sediments from various streams of Cauvery River, which is one of the major rivers of India (Begum et al., 2009).

HCH in soil

Lindane and other HCH isomers enter the soil compartment by direct application of the insecticide, by disposal of contaminated waste or by wet or dry deposition from the atmosphere. Once in the soil, HCHs are adsorbed to the soil particles, volatilized to the atmosphere, taken up by crop plants or leached into groundwater. HCHs adsorb strongly to organic matter (High K_{OW} values, Table 1) and are therefore relatively immobile in the soil. However, in soils with especially low organic matter content or subject to high rainfall, Lindane and other HCH isomers may pose a risk of groundwater contamination (Wauchope et al., 1992). Although both aerobic and anaerobic degradation of HCHs have been reported, they remain highly persistent in most soils. Depending on the conditions, field half-life values of a few days up to 3 years have been reported. Concentrations of the different HCH isomers vary from trace levels (<0.1 mg kg-1) (Albright et al., 1974) to < 1g kg^{-1} (WHO, 2005). Apart from diffuse pollution sources due to the application of

Lindane, a major problem is caused by point sources from remaining stockpiles of unused Lindane left behind all over the world (Vijgen et al., 2011). Furthermore, since the production of one ton of Lindane results in 8–12 tonnes of HCH-residuals (waste isomers), one may assume that millions of tonnes of this HCH-waste are still to be found all over the world. Concentrations of HCHs in soil in the vicinity of these kinds of stockpiles can reach peak concentrations in the range of grams per kg soil or mg per litre water (Vijgen, 2005). According to previous Indian industries production data, 56000 tonne stored/deposited HCH waste isomers are still present in the country (Vijgen et al., 2011). During the analysis of seasonal variation of HCH isomers in open soil and plant-rhizospheric soil system of a contaminated industrial area, Lucknow, India, Abhilash & Singh, (2009a) reported mean concentration of total HCH in plant samples, open soil and rhizospheric soil samples were found in the range of 14.12 to 59.29 mg kg^{-1}; 38.64 to 104.18 mg kg^{-1} and 8.38 to 26.05 mg kg^{-1}, respectively. In recent report, HCH contamination levels in soil and water samples collected around the production area and the vicinity of a major dumpsite were found to be very high (338,000 mg kg^{-1} of soil) as compared to HCH dumpsites/industrial waste sites from various other global locations (Jit et al., 2011).

HCH in food

Lindane and other isomers enter in the food either from soil, agricultural crops (sprayed with Lindane), etc. Under a multicentre study conducted by the Indian Council of Medical Research, 1712 samples of wheat grain/flour were collected from urban and rural areas in 11 states representing different geographical regions of India. Different

isomers of HCH were present in about 45-80% of the samples of wheat grain/flour with some of them above the maximum residue level value (Toteja et al., 2006). Earlier studies by Toteja et al., (2003) observed HCH residues in the 73% of 2000 rice samples collected from different geographical regions of India. Residues of HCH isomers were also found in milk, butter and ghee samples collected from different locations of India (Battu et al., 2004; Kumari et al., 2005). Recently Nag and Raikwar, (2011) reported contamination animal products like milk and meat with residues of HCH isomers and other organochlorine pesticides. In the samples of a highly contaminated area close to a small-scale industrial belt in Lucknow (North India), highest concentration of HCH isomers have been reported in the roots of plants compared to other parts, indicating that the most likely mechanism of HCH accumulation in these plants was sorption of soil HCH onto the roots (Abhilash et al., 2008).

MRLs values of Lindane

MRLs value for Lindane recommended by Codex Alimentarius Commision (Joint FAO/WHO Food Standards Programme) for various commodities such as Barley, Edible offal (mammalian), Eggs, Maize, Milks, Oats, Rye, Sorghum, Straw and fodder (dry) of cereal grains, Sweet corn (kernels), Poultry (Edible offal) and Wheat is 0.01, whereas in Meat (from mammals other than marine mammals-fat) and Poultry meat (fat) it is 0.1 and 0.05, mg/kg body wt, respectively(FAO/WHO, 2011).

Toxicological profile

Since, Lindane and other HCH isomers can be found in all environmental compartments, all organisms can come into contact with these pollutants. The effects of exposure depend on the exposure dosage, exposure duration, physiology of the organism, etc.

Effects on humans

Acute toxicity effects in humans can occur after ingestion of a certain amount of Lindane. Symptoms of acute toxicity in humans include headache, dizziness, seizures, diarrhoea, sickness and irritation of skin, nose, throat and lungs (Smith, 1991). Long-term exposure can cause chronic toxicity effects. In humans, chronic toxicity manifests itself as effects on the gastrointestinal tract, cardiovascular and musculoskeletal systems. Furthermore, HCHs can cause reproductive and endocrine effects by affecting the hormone production in ovaries and testes. Although, there is uncertainity about its mutagenicity and carcinogenicity, both USEPA and IARC consider Lindane and other HCHs as possible human carcinogens.

Effects on other organisms

Table 2 summarizes the toxicity of Lindane on a number of organisms in different the acute toxicity of isomers of HCH decreases in the order $\gamma < \alpha < \delta < \beta$. However, the toxicity of repeated doses decreases in the order $\beta < \alpha < \gamma < \delta$. The long-term toxicity of the different isomers is directly related to their adipose tissue storage and inversely related to their rate of metabolism (Hayes and Laws, 1991). Studies done on rats have

showed that HCH or other isomers adversely effects reproductive system, cardiovascular system, etc (Prasad et al., 1995; Anand et al., 1995).

Bioconcentration

An important toxicity aspect of HCHs, related to their high K_{ow} values, is the bioconcentration to high levels, following uptake from surface waters by a number of aquatic organisms. For example, bioconcentration factors (BCFs) in zebra-fish were 1100 for α-HCH, 1460 for β-HCH, 850 for γ-HCH, and 1770 for δ-HCH (Butte et al., 1991). Uptake from soils and bioconcentration by plants and terrestrial organisms appear to be limited. In tests with radiolabeled γ -HCH, grain, maize, and rice plants accumulated respectively 0.95, 0.11, and 0.04 % of the amount of bound γ-HCH residues following 14–20 days growth in a sandy loam soil. Bioconcentration increased by 4–10 times when the plants were grown in test soils containing both bound and extractable residues of γ-HCH (Verma and Pillai 1991). Uptake of γ-HCH by earthworm from soil has also been reported. Following exposure to 5 mg kg-1 of the compound for up to 8 weeks, the test organisms bioconcentrated γ-HCH by a factor of 2.5 (Viswanathan et al. 1988). Lindane and the other isomers of HCH do not appear to undergo biomagnification in terrestrial food chains to a great extent, although there is a moderate potential for transfer to animal tissue as a result of soil ingestion or ingestion of contaminated foliage (Wild and Jones 1992). For example, Clark et al., (1974) found that γ-HCH levels in the adipose tissue of cattle were 10 times higher than in the feed.

Table 2: Lindane concentrations showing toxic effects in different organisms (EXTOXNET, 1996; WHO, 2005)

Organism	Toxic effect	Value	Classification
Bacteria, algae, protozoa	NOEL[a]	1 mg l^{-1}	Slightly toxic
Fungi	NOEL	1-30 mg l^{-1}	Variable, slightly toxic
Bee	LD50[b]	0.56 µg bee^{-1}	Highly toxic
Fish, aquatic invertebrates	LC50[c]	2-90 µg l^{-1}	Highly to very highly toxic
Small rodents	LD50	60-500 mg kg^{-1} BW[d]	Moderately toxic
Birds	LD50	100->2000 mg kg^{-1} BW	Moderately to virtually nontoxic

[a] NOEL: No observed effect level: Concentration at which no noxious effects are observed
[b] LD50: Lethal dosage: Application dosage that causes a 50% mortality of a group of organisms
[c] LC50: Lethal concentration: Concentration in the medium that causes 50% mortality of the organisms placed in that medium (in this case after 96h)
[d] BW: Body weight

Mode of Action

Lindane is a contact insecticide with stomach and respiratory action. It acts as a stimulant to the nervous system causing epileptiform convulsions and death. In the nervous system, Lindane interferes with γ-aminobutyric acid (GABA) neurotransmitter function by interacting with the $GABA_A$ receptor-chloride channel complex at the picrotoxin binding site (ATSDR, 1997). Other effects include renal and liver damage. Lindane and ß-HCH interact with cellular membranes and may produce several generalized cytotoxic effects associated with impaired membrane function. For example, glucose uptake and cyclic AMP accumulation in rat renal cortical tubules were altered by exposure to Lindane (López-Aparicio et al., 1994).

Removal of Lindane (and other HCH isomers) from soil

Different approaches for removal (elimination) of Lindane and its waste isomers exist. Methods for removal of HCH involve physic-chemical methods and Bioremediation. The choice depends on the degree of urgency, type of contamination (in water, sediment or soil) and its concentration (ranging from the order of ng l^{-1} water or kg soil to almost pure product).

Physico-chemical techniques

Several physical-chemical techniques for the removal of Lindane and its waste isomers exist.A simple and obvious method would be combustion, but the need for very high temperatures and the additional need to strictly control reaction conditions in order to avoid the formation of undesirable toxic polychlorinated dioxins, make this technique

very expensive (Vikelsoe and Johansen, 2000). Some other physical-chemical non-combustion technologies for the removal of HCHs are listed in Table 3. Although very effective, these kinds of physical-chemical removal techniques are often very costly. Moreover, soil clean-up is often restricted to an *ex situ* treatment, which increases costs even more. These techniques are therefore economically only justified when dealing with very urgent and highly concentrated polluted sites.

Bioremediation

Microbial metabolism is accepted as a safer and efficient tool for the removal of many such organic pollutants. Bioremediation is the process by which living organisms degrade hazardous organic contaminants. The relatively inexpensive technology of bioremediation for reclaiming chemically contaminated land has therefore been steadily gaining acceptance since 1980's. Different bioremediation approaches have been successfully applied for the removal of soils contaminated with a variety of xenobiotic compounds (Newcombe and Crowley, 1999; Top et al., 1999; Cunningham and Philip, 2000; Juhasz et al., 2000; Runes et al., 2001; Manzano et al., 2003). Bioremediation may be applied after excavation of polluted site material and transport to a controlled environment (*ex situ*) or, under relatively natural conditions in the field (*in situ*).

***Ex-situ* Bioremediation**

In some cases, contaminated soils and water are best treated after being removed from the contamination site. This act of removing and then treating is known as *ex-situ* treatment.

Table 3: Physical-chemical non-combustion technologies for HCH removal from soil (USEPA, 2005)

Technology	Principle	*Ex / In situ*
Mechano-chemical dehalogenation	Mechanical energy is used to initiate chemical reactions. Intense mixing in a mill brings reactants into close contact. Grinding reduces particle size and increases surface area for reaction. New reactive surfaces are exposed and the introduction of dislocations increases surface reactivity. At contact between two grinding balls during a collision event, a highly localised triboplasma is formed giving energy for chemical reactions to occur. Milling also creates radicals, which can react with neighbouring compounds	*ex situ*
Gas-phase chemical reduction	Gas-phase chemical reduction of organic compounds by hydrogen at 850°C or higher. Chemical reduction to methane and hydrogen chloride. Efficiency is enhanced by the presence of water, which acts as a heat transfer agent and a source of hydrogen	*ex situ*
In situ thermal destruction	Heat and vacuum are applied to subsurface soils. Thermal conduction accounts for the majority of heat flow from the heaters (~800° C). Volatile and semi-volatile contaminants in the soil are vaporized and treated by a number of mechanisms: (1) evaporation into the subsurface air stream induced by application of vacuum, (2) steam distillation into the water vapor stream, (3) boiling, (4) oxidation, and (5) pyrolysis. The vaporized water, contaminants, and natural organic compounds are drawn by the vacuum in a counter-current direction to the heat flow into the vacuum source at the thermal wells or blankets	*in situ*
Base catalyzed decomposition	Heat treatment to 300°C in the presence of a reagent mixture consisting of a high boiling point hydrocarbon, sodium hydroxide and a proprietary catalyst. Highly reactive atomic hydrogen cleaves the chemical. The residues produced are carbon and sodium salts of anions liberated during the complete decomposition reactions, which are separated by gravity or centrifugation	*ex situ*

The most common processes of *ex-situ* treatment are pump-and-treat, biopile treatment and land farming. The pump-and-treat process is specifically designed to remediate contaminated groundwater. In this process, the contaminated water is removed from the ground and sent to an engineered treatment system. The treatment can occur via activated sludge, a trickling filter, rotating biological contactors, and ion exchange, among other possible systems (FRTR, 2006). Land farming, as other *ex-situ* treatment methods, involves the excavation and relocation of the contaminated media. The soil is tilled periodically to aerate the waste and remix the active microbes (Vaccari et al, 2006). In this process, several soil conditions are monitored to ensure a high rate of degradation. These criteria include, but are not limited to, moisture content, aeration and pH (FRTR, 2006). Throughout the bioremediation process, moisture and nutrients may be added to the soil as needed, and the pH can be controlled by adding crushed limestone or lime (FRTR, 2006). *Ex-situ* bioremediation generally requires a shorter amount of time to be completed than in-situ treatment (FRTR, 2006). Furthermore, *ex-situ* treatment is easier to control due to the possibility of continuous mixing during treatment. However, due to certain limitations of *ex-situ* bioremediation drastically affect the feasibility of using this technology. The *ex-situ* method requires excavation that increases the labor costs. Also in *ex-situ* methods a large amount of space is required for treatment. Volatile contaminants must be either contained or pretreated, as they may volatilize into the air if not monitored, and may cause air pollution (FRTR, 2006).

***In situ* Bioremediation**

Different bioremediation approaches for soil clean-up exist, including natural attenuation and enhanced natural attenuation (biostimulation or bioaugmentation).

Natural attenuation

The USEPA defines natural attenuation (NA) as the whole of processes of biodegradation, diffusion, dilution, sorption, volatilization and/or chemical and biological stabilization of contaminants, which effectively reduces toxicity, mobility or volume to levels that are protective of human health and the environment. These processes influence the persistence of soil contaminants, as is shown in Table 4 for persistent organic pollutants (POPs).Table illustrates the putative determinative role of microbial transformation in the process of NA processes of biodegradtion of POPs.

Microbial Biodegradation of Lindane (and other HCH isomers)

Various terminologies were used in bioremediations like biodegradation and mineralisation. *Biodegradation* is defined as "a process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment", whereas complete (bio)degradation to inorganic compounds is referred to as *mineralization.* In soil, microorganisms commonly exist in large populations. Provided with adequate supplies of carbon and energy and environmental conditions conducive to growth, microbial activity, especially the production of extracellular enzymes, can significantly assist in the amelioration of contaminated sites. Isolation of Lindane degrading microorganisms by enrichment culture has confirmed the ability of

Table 4: Natural attenuation processes that influence the persistence of POPs in soil (Peijnenburg, 2004)

NA processes in soil	Effect on POP persistence	Comment
Sorption	+++	Related to high K_{ow} values
Diffusion	+	Related low solubility
Dilution	+	Related low solubility
Volatilization	-	Even volatile POPs will volatilise only in limited amounts due to strong sorption to soil
Microbial transformation	+ → ++++	Major process (if present) that determines fate of POPs
Chemical/Biological stabilization	+++	Related to humification and ageing
Uptake by plants	-	Related to high lipophilicity
Uptake by soil fauna	++	Related to high bioconcentration factors

++++ : very general and strong; +++ : general and strong; ++ : regularly; + seldom/hardly; - : not

specific strains to degrade Lindane and other HCH isomers either aerobically or anaerobically. Anaerobic microbial degradation of HCH isomers was first reported in the 1960s, subsequently, aerobic bacteria capable of degrading HCH isomers were also found. Genes involved in degradation (*lin* genes) were isolated from aerobic bacteria, heterologously expressed, and the gene products have been characterized. Diversity, organization, and distribution of HCH catabolic genes has been studied particularly in sphingomonads (Lal et al., 2006; Nagata et al., 2007), Further bacteria from this genus were used to develop bioremediation technologies for the decontamination of HCH-contaminated sites (Bidlan et al., 2004; Böltner et al., 2007; Mertens et al., 2007; Raina et al., 2008).

Anaerobic HCH degradation

It was initially believed that HCH biodegradation is largely an anaerobic process and variable levels of anaerobic degradation of α-, β-, γ-, and δ-HCH have indeed been observed. Isolates capable of degrading one or more of the other four HCH isomers under anaerobic conditions listed in the Table 5. The γ- and α-isomers are degraded more rapidly than δ- and β-HCH (Datta et al., 2000; Johri et al., 1998; MacRae et al., 1967; Manonmani et al., 2000; Quintero et al., 2005). Several studies reported the production of chlorobenzene during the anaerobic degradation of γ-HCH (Figure 3) (Beland et al., 1976; Boyle et al., 1999; Middeldorp et al., 1996; Ohisa et al., 1980; Phillips et al., 2005). Ohisa et al. (1980) proposed that the degradation proceeds through two dichloroeliminations, resulting in the formation of γ-3,4,5,6-tetrachloro-1-cyclohexene

Figure 3: Anaerobic and aerobic degradation pathway of γ-HCH

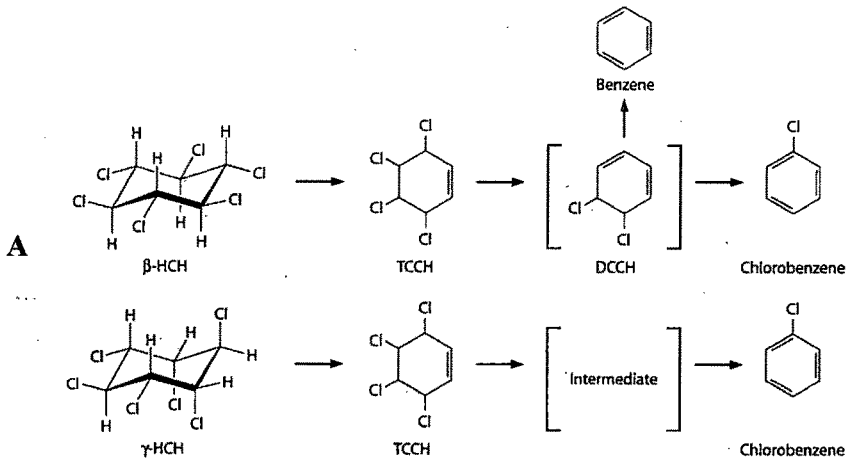
A. Consensus anaerobic degradation pathway of γ- HCH and β-HCH. Two intermediates that have been proposed but not yet observed empirically are shown in square brackets. The structures of TCCH and DCCH are shown in the planar format because their stereochemistries have not been established (Lal et al., 2011).

B. Proposed degradation pathways of γ-HCH in *S. japonicum* UT26 (Nagata et al., 2007).

Compounds:

1. γ-Hexachlorocyclohexane (γ-HCH),
2. Pentachlorocyclohexene (γ-PCCH),
3. 1, 3, 4, 6-tetrachloro-1, 4-cyclohexadiene (1, 4-TCDN),
4. 1, 2, 4-trichlorobenzene (1, 2, 4-TCB),
5. 2, 4, 5-trichloro-2, 5-cyclohexadiene-1-ol (2, 4, 5-DNOL),
6. 2, 5-dichlorophenol (2, 5-DCP),
7. 2, 5-dichloro-2, 5-cyclohexadiene-1, 4-diol (2, 5-DDOL),
8. 2, 5-dichlorohydroquinone (2, 5-DCHQ),
9. Chlorohydroquinone (CHQ),
10. Hydroquinone (HQ),
11. Acylchloride,
12. γ-hydroxymuconic semialdehyde,
13. maleylacetate (MA; 2-maleylacetate, 4-oxohex-2- enedioate),
14. β-ketoadipate (3-oxoadipate),
15. 3-oxoadipyl-CoA,
16. Succinyl-CoA,
17. Acetyl-CoA,
18. 2,6-dichlorohydroquinone (2,6-DCHQ), and
19. 2-chloromaleylacetate (2-CMA).

TCA (citrate/tricarboxylic acid cycle), **GSH** (glutathione-reduced form); **GS-SG** (glutathione-oxidized form). Square brackets show unstable compounds that are still undetected. The light and dark shaded areas indicate upstream and downstream pathways, respectively


A
Benzene
β-HCH
TCCH
DCCH
Chlorobenzene
γ-HCH
TCCH
Intermediate
Chlorobenzene

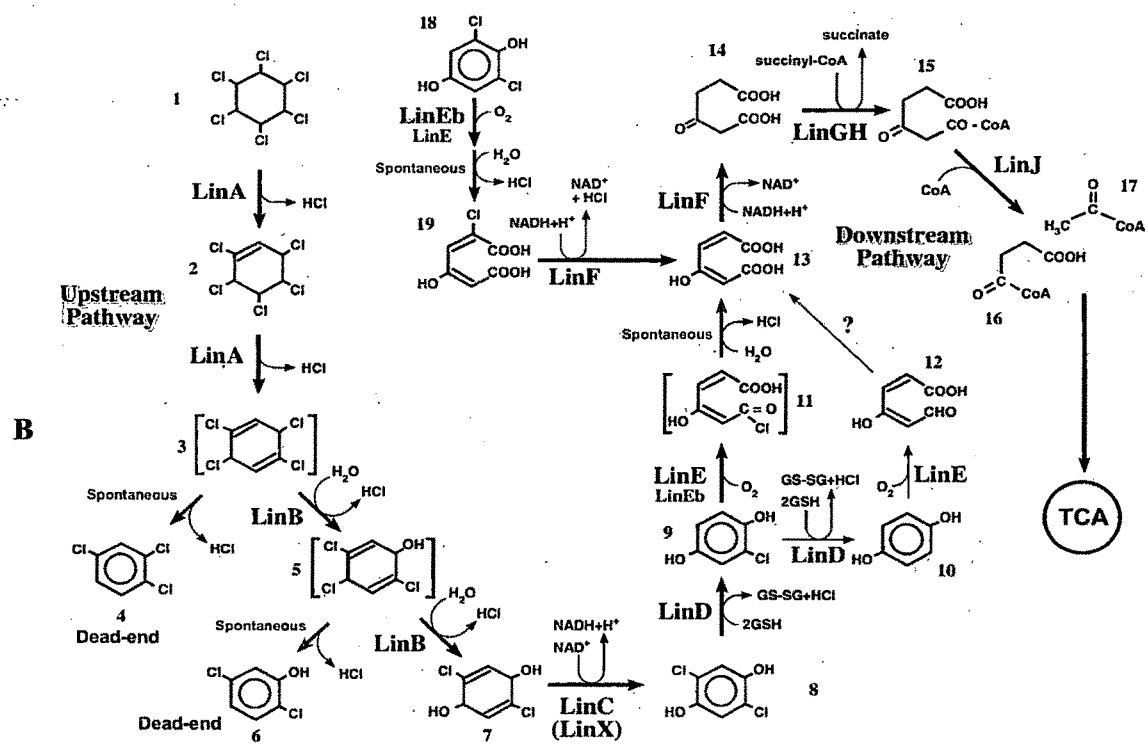

B
Upstream Pathway
LinA
LinA
LinB
LinB
LinC (LinX)
LinD
LinD
LinE LinEb
LinE
LinEb
LinF
LinF
LinGH
LinJ
Downstream Pathway
Spontaneous
Spontaneous
Spontaneous
Dead-end
Dead-end
succinyl-CoA
succinate
CoA
CO - CoA
GS-SG+HCl
2GSH
NADH+H+
NAD+
TCA

Table 5: List of microorganisms reported for HCH biodegradation

A. Isolates capable of degrading one or more of the other four HCH isomers under anaerobic conditions

B. Isolates capable of degrading one or more of the other four HCH isomers under aerobic conditions

A.

Clostridium rectum	Ohisa et al., 1978
Clostridium sphenoides	Heritage et al., 1977, MacRae et al., 1969
Clostridium butyricum, Clostridium pasteurianum, Citrobacter freundii	Jagnow et al., 1977
Desulfovibrio gigas, Desulfovibrio africanus, Desulfococcus multivorans	Boyle et al., 1999
Dehalobacter sp.	Van et al., 2005

B.

Organism(s)	Reference(s)
Alcaligenes faecalis S-1 and *A. faecalis* S-2	Gupta et al., 2001
Arthrobacter citreus BI-100	Datta et al., 2000
Bacillus circulans and *Bacillus brevis*	Gupta et al., 2000
Bacillus sp.	Yule et al., 1967
Escherichia coli	Francis et al., 1975
Microbacterium sp. ITRC1	Manickam et al., 2006b
Pandoraea sp.	Okeke et al., 2002
Pseudomonas aeruginosa	Lodha et al., 2007
Pseudomonas aeruginosa ITRC-5	Kumar et al., 2005
Pseudomonas putida	Benezet et al., 1973
Pseudomonas sp.	Nawab et al., 2003
Pseudomonas sp.	Tu, 1976
Rhodanobacter lindaniclasticus RP5557173	Thomas et al., 1996
Sphingobium chinhatense IP26	Dadhwal et al., 2009b
Sphingobium indicum B90A	Sahu et al., 1990
Sphingobium japonicum UT26	Senoo and Wada, 1989
Sphingobium francense Sp+	Cérémonie et al., 2006
Sphingobium quisquiliarum P25	Bala et al., 2009
Sphingobium ummariense RL^{-3}	Singh and Lal, 2009
Sphingobium sp. BHC-A	Ito et al., 2007
Sphingobium sp. MI1205	Ma et al., 2005
Sphingobium sp. (SS04-1, SS04-2, SS04-3, SS04-4, SS04-5)	Yamamoto et al., 2009
Sphingobium sp. (UM1, UM2, HDU05, HDIP04, F2)	Dadhwal et al., 2009a
Sphingomonas sp. (DS2, DS2-2, DS3-1)	Böltner et al., 2005
Sphingomonas sp. (α1-2, α4-2, α4-2, α4-5, α16-10, α16-12, γ1-7, γ12-7, γ16-1, γ16-9	Mohan et al., 2006
Streptomyces sp. M7	Benimeli et al., 2006
Xanthomonas sp. ICH12	Manickam et al., 2007

(γ-TCCH) and, subsequently, 5,6-dichlorocyclohexa-1,2-diene. Chlorobenzene is then produced by a dehydrochlorination reaction. Several reports confirmed the presence of γ-TCCH as an intermediate in the anaerobic degradation of γ-HCH (Beland et al., 1976; Heritage et al., 1977; Jagnow et al., 1977; Ohisa et al., 1980). Furhter there are reports which suggest that the anaerobic degradation of α-, β-, and δ-HCH also proceeds at least in part through successive dichloroeliminations and then dehydrochlorination to produce chlorobenzenes. Recent studies have confirmed that β-HCH can be converted to both benzene and chlorobenzene under anaerobic conditions (Middeldorp et al., 2005; Van et al., 2005). Quintero et al. (2005) suggested that another anaerobic pathway could also generate chlorobenzene from all four of the major HCH isomers. They studied anaerobic liquid and soil slurry systems of undefined microbial contents and observed the production of pentachlorocyclohexane, followed by 1, 2- and 1, 3-dichlorobenzene and then chlorobenzene. For the α- and γ-isomers, these authors also observed the intermediate products tetrachlorocyclohexene and trichlorobenzene. One major factor restricting the microbial degradation of HCH isomers under anaerobic conditions is the strong adsorption of the isomers onto soil (Rijnaarts et al., 1990); degradation is much faster under liquid or slurry conditions (Doelman et al., 1985; Quintero et al., 2005; Rijnaarts et al., 1990). Most studies of anaerobic HCH degradation reported the accumulation of chlorobenzene and benzene (Buser et al., 1995; Middeldorp et al., 1996; Eekert et al., 1998), although Quintero et al., (2005) detected only traces of these metabolites to chloride and chloride-free metabolites in mixed and pure cultures of *Clostridium* species. Furhter, there are reports of the anaerobic mineralization of benzene (Edwards et al., 1992; Varga et al., 1999). Both chlorobenzene and benzene, however,

can be also readily mineralized under aerobic conditions (Deeb et al., 2000; Deeb et al., 1999; Fairlee et al., 1997; Mars et al., 1997).

Aerobic HCH degradation

Several studies have reported various levels of degradation of the four major HCH isomers under aerobic conditions (Bachmann et al., 1988; Nagasawa et al., 1993; Sahu et al., 1992, 1993 and 1995). Most of the HCH-degrading aerobic bacteria known to date are members of the family *Sphingomonadaceae* (Lal et al., 2008; Lal et al., 2006); around 30 HCH-degrading sphingomonads have been reported from different geographical areas (Table 5). The first strain was reported in the late 1980s for HCH degradation from Japan (Senoo et al., 1989). Most research has focused on *Sphingomonas paucimobilis*, strain UT26, B90A, and Sp+, isolated from HCH contaminated soils in Japan (Senoo et al., 1989), India (Sahu et al., 1990), and France (Cérémonie et al., 2006), respectively. A polyphasic approach including 16S rRNA gene analysis subsequently revealed that these strains *Sphingomonas paucimobilis* UT26, B90A and Sp+ are actually three distinct species of *Sphingobium*: *Sphingobium japonicum* UT26, *Sphingobium indicum* B90A, and *Sphingobium francense* Sp+, respectively (Pal et al, 2005). Furhter reports in past few years shown of γ-HCH and/or other isomers degraders from India (Kumar et al., 2005; Manickam et al., 2008). Although all these strains degrade HCH, isomer-specific differences between strains in the early steps of the degradation pathway have been (Böltner et al., 2005; Nagata et al., 2007; Lal et al., 2010).

γ-HCH biodegradation

The aerobic degradation pathway of γ-HCH has been studied in considerable detail in *S. japonicum* UT26 (Nagata et al., 2007; Nagata et al., 1999b) (Figure 3). It was suggested that two initial dehydrochlorination reactions produce the putative product 1, 3, 4, 6-tetrachloro-1,4-cyclohexadiene (1,3,4,6-TCDN) via the observed intermediate γ-pentachlorocyclohexene (γ-PCCH) (Imai et al., 1991). Subsequently 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) is generated by two rounds of hydrolytic dechlorinations via a second putative metabolite, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL) (Nagata et al., 1993c). 2,5-DDOL is then converted by a dehydrogenation reaction to 2,5-dichlorohydroquinone (2,5-DCHQ) (Nagata et al., 1994). The formation of 2,5-DCHQ completes what is known as the upstream degradation pathway. It was suggested that the major upstream pathway reactions described above are enzymatically catalyzed, but two other, minor products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol (2,5-DCP), are produced, presumptively by spontaneous dehydrochlorinations of the two putative metabolites, 1,3,4,6-TCDN and 2,4,5-DNOL (Nagasawa et al., 1993a, 1993b, 1993c). Both 1,2,4-TCB and 2,5-DCP appear to be dead-end products in this strain. The first step in the subsequent, downstream degradation pathway is a reductive dechlorination of 2,5-DCHQ to chlorohydroquinone (CHQ) (Miyauchi et al., 1998). The pathway then bifurcates, with the minor route being a further reductive dechlorination to produce hydroquinone (HQ), which is then ring cleaved to γ-hydroxymuconic semialdehyde (γ-HMSA). The major route involves the direct ring cleavage of CHQ to an acylchloride, which is further transformed to maleylacetate (MA) (Miyauchi et al., 1999). MA is converted to β-ketoadipate (Endo et al., 2005) and then to

succinyl coenzyme A (CoA) and acetyl-CoA, which are both metabolized via citric acid cycle (Nagata et al., 2007). Recently novel metabolite like 2,5-dichlorobenzoquinone (2,5-DCBQ) was observed after the degradation 2,5 dichloro-cyclohexadiene-1,4-diol (2,5-DDOL) in *Xanthomonas* sp. ICH12 (Manickam et al., 2007).

Degradation of other isomers

α-HCH actually exists in two enantiomeric forms, each of which is converted to its respective β-PCCH enantiomer, these two β-PCCHs are then metabolized to 1, 2, 4-TCB (Saur et al., 2005). β-HCH is the most recalcitrant of all the major HCH isomers and does not undergo mineralization easily (Bachmann et al., 1988). Its relative stability is attributed to the fact that it is the only fully equatorially substituted HCH isomer (Figure 1). This appears to be a barrier to the dehydrochlorination reactions, which are the first steps in the degradation of γ- and α-HCH described above and which apparently require axial chlorine atoms. Instead, it seems that hydrolytic dechlorination reactions now become more feasible. The transformation of β-HCH to 2,3,4,5,6-pentachlorocyclohexanol (PCHL) has been observed for all five strains tested . PCHL appears to be a terminal product in *S. japonicum* UT26 and *S. francense* Sp+ (Nagata et al., 2005; Sharma et al., 2006), whereas in *S. indicum* B90A, *Sphingobium* sp. MI1205, and *Sphingobium* sp. BHC-A, it is further converted to 2,3,5,6-tetrachlorocyclohexanediol (2,3,5,6-TCDL) (Ito et al., 2007; Raina et al., 2007; Wu et al., 2007a). Therefore, differences between strains have been observed in the metabolism of β-HCH. In *Sphingobium* sp. strain BHC-A (Wu et al., 2007b) reported that LinA catalyses dehydrochlorination of 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 can 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).

Biodegradation by fungi

Pure cultures of the lignin-degrading fungi *Phanerochaete chrysosporium* and *Trametes hirsutus* have been shown to degrade Lindane (Bumpus et al., 1985; Kennedy et al., 1990; Mougin et al., 1997; Singh and Kuhad, 1999). The proposed mechanism for degradation was similar to that of lignin degradation, i.e. multiple non-specific oxidative reactions resulting from generation of carbon-centered free-radicals (Bumpus et al., 1985). The ability of several white rot fungi (*Pleurotus sajorcaju, Pleurotus florida and Pleurotus eryngii*) to degrade Lindane was reported by Arisoy (1998).

Factors affecting HCH biodegradation

Many variables affect the rate of biodegradation in soil which may vary from location to location for a given set of environmental conditions. These variables can be split up in three different groups: microbiota related, substrate related and soil related factors.

Redox potential

Since HCH biodegradation was initially thought to be an anaerobic process (MacRae et al., 1967; Siddaramappa and Sethunathan, 1975; Jagnow et al., 1977; MacRae et al., 1984; Chessels et al., 1988), anoxic conditions were suspected to increase HCH removal rates. Chessels et al. (1988) reported a correlation between soil moisture content and the degradation of HCHs. Aerobic conditions may however exist at the water-air interphase (Siddaramappa and Sethunathan, 1975), therefore the assessment of the redox potential is important since it is now known that aerobic biodegradation also occurs. Doelman et al., (1990) reported the degradation of α-HCH under oxic conditions, while Bachmann et al., (1988) studied the degradation of α-HCH under oxic, denitrifying, sulphate-reducing and methanogenic conditions in soil slurries. In the latter study it was shown that degradation was most rapid under oxic conditions, although significant reduction was observed under methanogenic conditions. Denitrifying and sulphate-reducing conditions did not establish significant degradation of α-HCH. Middeldorp (1996) reported the degradation of α-HCH under methanogenic conditionsand also showed the degradation of β-, γ- and δ-HCH under these conditions, providing evidence of the removal of these isomers under anoxic conditions. MacRae (1984), on the other hand, showed that all four isomers could also be degraded aerobically. Evidence of aerobic γ-HCH removal was also provided by Bhuyan et al., (1992) and Yule et al., (1967). β-HCH is generally accepted to be the most recalcitrant HCH isomer (Bachmann et al., 1988; Doelman et al., 1990), and several studies have suggested that significant β-HCH degradation does not occur under oxic conditions (Siddaramappa and Sethunathan, 1975; Van Eekert et al., 1998). MacRae (1984) reported 14% β-HCH removal from soil slurry, whereas Phillips et al., (2000)

showed 11% removal of β-HCH in soil microcosms, under oxic conditions. Recently, several bacteria of the genus *Sphingomonas* have been reported to be able to aerobically degrade β-HCH (Suar et al., 2004; Böltner et al., 2005). Moreover, complete mineralization of HCHs is generally only reported under oxic conditions (Bachmann et al., 1988; Huntjens et al., 1988; Sahu et al., 1990; Nagasawa et al., 1993).

Bioavailability

The same factors that affect solubility and sorption of HCHs influence their movement within the soil matrix, thereby affecting their bioavailability and biodegradation. Although organic matter increases the adsorption of organic contaminants to soil and as such reduces their bioavailability (El Beit et al., 1981), it also might increase microbial activity and thus enhance biodegradation rates. Other factors that were shown to increase adsorption of HCHs in soil are clay content and iron and magnesium minerals (El Beit et al., 1981). On the other hand, Chessels et al. (1988) indicated that higher silt content results in higher moisture retention, which in turn increases bioavailability by inhibiting sorption.

Temperature and pH

Optimum pH and temperature ranges were studied for several HCH-degrading species. For a *Pandoraea* species (Okeke et al., 2002) optimal degrading conditions in soil were determined to be pH 9.0 and 25-30°C. *Clostridium rectum* had an optimum at a pH of 7-8 and a temperature 37-38°C. In recent report it has been showed that *Sphingobium* strains (*S. indicum* B90A, *S. japonicum* UT26 and *S. francense* Sp+) were able to degrade γ-

HCH under low temperature at 4^0C (Zheng et al., 2011). It has however been shown in some studies that the influence of temperature is not as significant as that of other soil conditions such as redox conditions and moisture content (Doelman et al., 1988a, b).

Contaminant HCH) concentration

Biodegradation rates in soil generally follow first-order kinetics and are concentration dependent. As a consequence, HCH removal rates might be expected to slow down as the HCH concentrations decrease (El Beit et al., 1981).

Lin genes involved in biodegradation of HCH

Genes necessary for the aerobic degradation of γ-HCH (called *lin* genes) were initially identified and characterized from *S. japonicum* UT26 (Nagata et al., 1999b) (Figure 3) (Table 6) and subsequently reported in varius other γ-HCH degraders (Kumari et al., 2002; Dogra et al., 2004; Singh et al., 2007a; Manickam et al., 2008). *lin*A to *lin*C encode the enzymes responsible for the upstream pathway, and *lin*D to *lin*J encode those enzymes for the downstream pathway.

Enzymes involved in upstream pathway

Evidence across a variety of strains indicates that the *linA*-encoded HCH dehydrochlorinase (LinA) (Raina et al., 2007; Saur et al., 2005; Wu et al., 2007) and the *linB*-encoded haloalkane dehalogenase (LinB) (Ito et al., 2007; Nagata et al., 2005;

Table 6: HCH-degradative (*lin*) genes in HCH-degrading *Sphingomonads* (Lala et al., 2010)

Gene	Amino acid residue(s)	Function	Strains (GenBank accession no.)
linA1/linA/linA2	154/156	Dehydrochlorinase	UT26 (D90355), B90/B90A (AY331258, AY150580), Sp+ (AY903217, AY690622), DS2 (AJ871378), DS2-2 (AJ871379), γ1-7 (AJ871380), δ12-7 (AJ871381), γ16-1 (AJ871382), α1-2 (AJ871383), α4-2 (AJ871384), DS3-1 (AJ871385), BHC-A (DQ372106)
linB	296	Haloalkane dehalogenase	UT26 (D14594), B90A (AY331259), Sp+ (AY903216), BHC-A (DQ246619), MI1205 (AB278602), SS04-1 (AB304077), SS04-2 (AB304078), SS04-3 (AB304079), SS04-4 (AB304080), SS04-5 (AB304081)
linC	250	Dehydrogenase	UT26 (D14595), B90A (AY331258), Sp+ (DQ111068), BHC-A (DQ462464)
linD	346	Reductive dechlorinase	UT26 (D89733), B90A (AY334273), BHC-A (DQ480725)
linE	321	Ring cleavage oxygenase	UT26 (AB021867), B90A (AY334273), BHC-A (DQ399709)
linR	303	LysR family transcriptional regulator	UT26 (AB021863), B90A (AY334273), BHC-A (DQ399711)
linF	352	Maleylacetate reductase	UT26 (AB177985), BHC-A (DQ399710)
linEb	319	Ring cleavage oxygenase	UT26 (AB177985)
linG	156	Acyl-CoA transferase	
linH	212	Acyl-CoA transferase	
linI	267	IclR family transcriptional regulator	
linJ	403	Thiolase	
linK	316	Putative ABC transporter system, inner membrane protein	UT26 (AB267475)
linL	282	Putative ABC transporter, ATPase	UT26 (AB267475)
linM	320	Putative ABC transporter system, periplasmic protein	UT26 (AB267475)
linN	202	Putative ABC transporter system, lipoprotein	UT26 (AB267475)
linX[b]	250	Dehydrogenase	UT26 (D23722), B90/B90A (AY331258, AY150580), BHC-A (DQ486136)

Raina et al., 2007; Sharma et al., 2006; Wu et al., 2007a and 2007b) catalyze the dehydrochlorinase and hydrolytic dechlorinase reactions, respectively, in the upstream pathway. There is unequivocal experimental evidence that LinA dehydrochlorinates γ-, α-, and δ-HCH, while LinB hydrolytically dechlorinates β- and δ-HCH in all strains examined. However, an interpretation of the roles of LinA and LinB in some of the subsequent reactions is not so straightforward because certain key metabolites have not been recovered experimentally for the dehydrochlorinase-led pathway for the γ-, α-, and δ-isomers, and there are strain differences with respect to the hydrolytic dechlorinase-led pathway for β- and δ-HCH.

LinA (HCH dehydrochlorinase)

LinA is a homotetrameric protein with a 16.5-kDa molecular mass (Nagata et a., 1993a and 1993b), which appears to be located in the periplasm of sphingomonads (Nagata et al., 1999a), although a homologue in *Rhodanobacter lindaniclasticus* appears to be secreted extracellularly (Nalin et al., 1999; Thomas et al., 1996). LinA is believed to be a unique type of dehydrogenase. It has no close relatives but shows low-level sequence similarity to a small group of proteins of diverse function with an α/β-crystatin-like structural fold (Nagata et al., 2001, Trantirek et al., 2001). Only qualitative work on the substrate range of LinA has been performed so far (Nagata et al., 1993a), and this suggests that its substrate range may be restricted to α-, γ-, and δ-HCH and their corresponding PCCH products. Since β-HCH, which is not hydrolyzed by LinA, is the only major isomer that lacks at least one adjacent *trans*-diaxial hydrogen/chlorine pair (Figure 1), it was proposed and subsequently confirmed that the dehydrochlorination of

the other HCH isomers by LinA occurs stereoselectively at this H/Cl pair (Nagasawa et al., 1993c; Nagata et al., 1993a; Trantirek et al., 2001). A similar reaction mechanism was proposed for the PCCHs, yielding TCDNs (Nagata et al., 1993a; Trantirek et al., 2001; Wu et al., 2007a). However, Wu et al. (2007b) reported that LinA catalyzes the dehydrochlorination of δ-HCH and its product, δ-PCCH, even though δ-PCCH does not possess a *trans*-diaxial H/Cl pair.

Most strains tested have one copy of the *linA* gene, but *S. indicum* B90A and *Pseudomonas aeruginosa* ITRC-5 are known to have two, denoted as *linA1/linA2* and *linAa/linAb* (Dogra et al., 2004; Kumari et al., 2002; Lal et al., 2006; Singh et al., 2007a). There is also evidence from DNA hybridization studies that some other strains may also have two copies of the *linA* gene (Dadhwal et al., 2009a). The LinA1 and LinA2 enzymes from *S. indicum* B90A are known to differ by about 10% in their amino acid sequences. The *linA* genes of *S. japonicum* UT26 and *S. indicum* B90A were shown to be constitutively expressed (Nagata et al., 1999b; Saur et al., 2004), although there is evidence for inducible expression of *lin*A in *Rhodanobacter lindaniclasticus* (Nalin et al., 1999; Thomas et al., 1996).

LinB (haloalkane dehalogenase)

LinB is a monomeric 32-kDa protein (Nagata et al., 1993c), which, like LinA, is located in the periplasm of the sphingomonads (Nagata et al., 1999a). The major catalytic domain of LinB belongs to the large and well-characterized α/β-hydrolase fold superfamily of proteins, which characteristically carry out two-step hydrolytic reactions driven by a

nucleophile (Asp) that is part of a catalytic triad, using an oxanion hole to stabilize the intermediate (Nagata et al., 1993c and 1997; Kmunicek et al., 2005). The specificity of LinB for different HCH isomers has not been studied in any detail. However, data for purified, heterologously expressed LinB (Kmunicek et al., 2005) reveal a very broad substrate preference for halogenated compounds up to a chain length of eight carbon atoms. The maximum catalytic efficiency (k_{cat}/K_m) was found to be 233 s^{-1} mM^{-1} for 1-iodohexane. LinB was also shown to catalyze the hydrolytic dechlorination of β- and δ-HCH although at considerably lower efficiencies (k_{cat}/K_m = 1 s^{-1} mM^{-1} for β-HCH) (Nagata et al., 2005). Heterologously expressed LinB does not catalyze the hydrolytic dechlorination of γ-HCH, although, low-level activity against the α-isomer was recently reported for LinB from *S. indicum* B90A (Raina et al., 2008). Significantly, LinB from *S. indicum* B90A and *Sphingobium* sp. MI1205 is an order of magnitude more efficient than LinB from *S. japonicum* UT26 at catalyzing the dechlorination of β-HCH, and it also catalyzes the dechlorination of the product PCHL at the 4-position, resulting in the dihydroxylated product 2,3,5,6-TCDL (Ito et al., 2007; Sharma et al., 2006). The biochemical differences between LinB of *S. indicum* B90A and LinB of *S. japonicum* UT26 provide an elegant explanation for the differences between these two strains in their performance of the hydrolytic dechlorination-led degradation of β-HCH.

Interestingly, while most HCH-degrading strains have only a single *linB* gene, *Sphingobium* sp. *Sphingobium* sp. MI1205 and *P. aeruginosa* ITRC-5 were reported to contain an IS*6100*-flanked duplication of a *linB* variant similar to the *linB* sequences found in strains *S. indicum* B90A and *Sphingobium* sp. BHC-A (Ito et al., 2007; Singh et

al., 2007a). This cluster is also identical to the one found for plasmid pLB1, isolated from soil, suggesting a horizontal transfer of *lin* genes (Miyazaki et al., 2006). The *linB* gene is known to be constitutively expressed in *S. japonicum* UT26 and *S. indicum* B90A at least (Nagata et al., 1999b; Saur et al., 2004). Raina et al. (2008) showed that resting cells of *E. coli* heterologously expressing the *linB* gene from *S. indicum* B90A can act on α-HCH and on β- and γ-PCCH. Two stereoisomers of 3,4,5,6-tetrachloro-2-cyclohexene-1-ol and 2,5,6-trichloro-2-cyclohexene-1,4-diol were identified as being products of these reactions. These metabolites have not been reported for HCH-degrading strains, and α-HCH had not previously been reported as a substrate for any LinB variant. Moreover, the kinetics of these reactions were not reported by Raina et al., (2008).

LinC

LinC is a 2, 5-DDOL dehydrogenase in the short-chain alcohol dehydrogenase family (Nagta et al., 1994; Persson et al., 1991). A general catalytic mechanism was proposed for these enzymes (Jornvall et al., 1995), involving the participation of a conserved Ser-Tyr-Lys catalytic triad and an NAD^+ cofactor. Specifically, the tyrosine hydroxyl is stabilized in a deprotonated state by the amino group of the lysine. The deprotonated tyrosine then participates in proton abstraction from the hydroxyl group of the substrate, which is further activated through a hydrogen-bonding interaction with the serine. Finally, hydride transfer from the substrate to NAD^+ forms NADPH and the reduced product, i.e., the conversion of 2,5-DDOL to 2,5-DCHQ. *linC* genes have been recovered from several HCH-degrading sphingomonads (Böltner et al., 2005, Cérémonie et al., 2006; Dogra et al., 2004; Nagta et al., 1994; Wu et al., 2007a), as a single copy in both

S. japonicum UT26 and *S. indicum* B90A (Dogra et al., 2004; Nagta et al., 1994) but in two copies in *S. francense* Sp+ (Lal et al., 2006). Variants of LinC have also been amplified directly from HCH-contaminated soils (Genki et al., 2008). Amino acid identities among the available sphingomonad LinC sequences are 98 to 100%. Like *linA* and *linB*, *linC* was found to be constitutively expressed in strains *S. japonicum* UT26 and *S. indicum* B90A (Nagata et al., 1999b; Saur et al., 2004). More divergent (97 to 98% amino acid identities) *linC* genes have also been recovered from HCH-degrading *Pseudomonas aeruginosa* ITRC-5 (Singh et al., 2007a) and *Microbacterium* sp. ITRC1 (Manickam et al., 2006). Nagata et al., (1994) also discovered a *linX* gene in the vicinity of the *linA* gene of *S. japonicum* UT26 that encodes a protein with 33% amino acid identity to LinC and also some limited 2,5-DDOL dehydrogenase activity. *linX* genes have now been found in most of the HCH-degrading sphingomonads, generally in multiple copies (Böltner et al., 2005; Dogra et al., 2004). Three copies are found in the vicinity of the *linA1/linA2* genes in *S. indicum* B90A; *linX1* and *linX3* are identical, and *linX2* is 67% different at the amino acid level (Dogra et al., 2004). Nagata et al., (2007) showed that LinX is not necessary for the degradation of γ-HCH *in vivo*, but its real physiological role is not clear.

The downstream pathway

Most of the HCH-degrading sphingomonads tested, with the notable exception of *Sphingomonas* sp. γ12-7, contain the genes *linD* and *linE*, which encode the first two (reductive dechlorinase and ring cleavage oxygenase, respectively) steps in the downstream pathway, as well as the regulatory gene *linR*, which encodes the LysR-type

transcriptional regulator LinR (Böltner et al., 2005; Cérémonie et al., 2006; Dogra et al., 2004; Nagata et al., 1999b; Wu et al., 2007b). These three genes as well as *linF* (encoding maleylacetate reductase) are also present in the HCH-degrading *Pseudomonas aeruginosa* ITRC-5 (Singh et al., 2007a). In strain *S. japonicum* UT26, the *linD* and *linE* genes are located near each other and may form an operon (Miyauchi et al., 1999), and their transcription is induced by LinR in the presence of their respective substrates 2,5-DCHQ (LinD), CHQ (LinD), and HQ (LinE) (Miyauchi et al., 2002) (Figure 3). Further, Suar et al. (2004) reported that the *linD* and *linE* genes can also be induced with α- and γ- but not β- and δ-HCH in strain *S. indicum* B90A; as noted above, this strain will produce the 2,5-DCHQ, CHQ, and HQ substrates for LinD and LinE from γ- and δ-HCH. Strain *S. japonicum* UT26 also has a second ring cleavage oxygenase, LinEb, which is 53% identical to LinE and also contributes to the degradation of CHQ (Endo et al., 2005). The *linEb* gene is located close to the *linF* gene, and an open reading frame located between the two encodes another LysR-type regulator, which is implicated in their regulation (Endo et al., 2005).

The *linGH* and *linJ* genes, which encode the acyl-CoA transferase and thiolase required for the conversion of β-ketoadipate to succinyl-CoA and acetyl-CoA, were recently identified in *S. japonicum* UT26, as was the *linI* gene, which encodes an IclR-type transcriptional regulator that may regulate their expression (Nagata et al., 2007).The *linK*, *linL*, *linM*, and *linN* genes recently identified in *S. japonicum* 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 γ-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. The organization and diversity of *lin* genes have been studied among several sphingomonads, and they have been found to be associated with plasmids and IS6100, both of which appear to have a significant role in horizontal gene transfer (Lal et al., 2006).

Enhanced natural attenuation

When human intervention occurs in order to stimulate biodegradation it leads to enhanced natural attenuation. Various degrees of interventions may be implemented. Certain compounds can be added to the soil in order to improve environmental conditions that stimulate biodegradation. This is referred to as biostimulation. The actual addition of specific degrading microorganisms is called bioaugmentation.

Biostimulation

One approach of bioremediation is the optimization of the environmental conditions to stimulate growth and biodegradation of HCHs by indigenous microorganisms. Supplementation of nutrients and organic amendments may be done to enrich the habitat for degrading organisms. Inorganic macronutrient sources of nitrogen, phosphorus, magnesium or potassium may be added to stimulate degradation. For example, MacRae (1984) showed a stimulated removal of α-, β- and δ-HCH by the amendment of urea.

Potassium chloride and potassium sulphate were also shown to enhance γ-HCH degradation in cell suspensions of *Clostridium* sp. (Sethunathan et al., 1969). Peptone, yeast extract are often required in the degradation of HCH by cultures that are unable to metabolize HCHs without another source of carbon in the media (Ohisa et al., 1980; MacRae et al., 1984; Maule et al., 1987). Other carbon sources might enhance degradation in soil where carbon is lacking, but will inhibit HCH removal when added in excess (Castro and Yoshida, 1974; Siddaramappa and Sethunathan, 1975; Sahu et al., 1993). Plant-derived organic amendments are often used in bioremediation because of the availability of large quantities of raw material and low cost. They are selected on the basis of desirable C:N:P ratios to be used as nutrient source, but they also improve aeration and water retention in the soil (Castro and Yoshida, 1974; Siddaramappa and Sethunathan, 1975; Bhuyan et al., 1993; Manonmani et al., 2000; Phillips et al., 2005).

Bioaugmentation

Another approach of bioremediation of xenobiotic chemicals is bioaugmentation, i.e. the inoculation of specialized degrading microorganisms, in order to introduce competent bacteria and minimize the lag period before biodegradation begins. Inoculation of contaminated soil with acclimated microorganisms derived from similar contaminated environments might result in enhanced biodegradation. Another possibility is the inoculation of pure degrading strains, that have been isolated from another source or that have been genetically modified (GMOs).

An important factor that may determine the success of bioaugmentation is the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat. Once applied, the success of a bioaugmentation procedure depends on the establishment of microorganisms and on the expression of the necessary degradative genes (Daubaras and Chakrabarty, 1992; Providenti et al., 1993). In general, population sizes of bacteria decline more or less rapidly following introduction into a natural soil. Also the growth of introduced bacterial populations in microbiologically undisturbed soils is a rare phenomenon. This growth/survival-inhibitory effect of soil has been called microbiostasis (Ho and Ko, 1985). It is attributed to the scarcity of available nutrient sources and the hostility of the soil environment to incoming microorganisms due to both abiotic and biotic factors. Thus, field studies or microcosum studies could be an alternative to analyse the suitability of isolate in the soil.