SCREENING AND ISOLATION OF γ-HEXACHLOROCYCLOHEXANE DEGRADING BACTERIA FROM CONTAMINATED SOIL IN KENYA

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DECLARATION

I hereby declare that this thesis is my original work and that it has not been presented for a degree in any other University.

Signature……

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LIST OF ABBREVATIONS

ASTRD	Agency for Toxic Substances and Disease Registry
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl Trimethylammonium Bromide
DCM	Dichlorometnane
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
GC-MS	Gas Chromatography- Mass spectrophotometry
HCH	Hexachlorocyclohexane
NCBI	National Center for Biotechnology information
PCR	Polymerase Chain reaction
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
TE	Tris- Ethylenediaminetetraacetic acid
UV	Ultra Violet

ABSTRACT

The organochlorine insecticide Lindane is the γ-isomer of hexachlorocyclohexane (HCH). It is among the most distributed and frequently detected organochlorine pollutants in the environment. Consequently it has been flagged down for regulatory intervention and efforts are being made to eliminate it from the environment. Among the approaches proposed, bioremediation has been put forward as a promising environmental friendly approach. In line with this mission, the aim of this work was to isolate and identify bacteria able to utilize Gamma-1, 2,3,4,5,6-hexachlorocyclohexane (γ-HCH) as a sole carbon source, and thus potential bioremediation agents. Using soil sample collected from a site contaminated with organochlorine pesticide, three isolates identified by 16S rRNA gene sequencing as *Achromobacter* sp. (isolate 1 and isolate 3) and *Stenotrophomonas* sp. (isolate 2) were observed to grow in minimal salt media containing γ-HCH as the sole carbon. They were all observed to be gram-negative rods that formed circular convex shaped colonies on nutrient agar. Their optimal growth temperature and pH was found to be 37 ^{o}C and 7 respectively. Growth of these isolates in minimal salt media containing γ-HCH as the sole carbon source after 10 days indicated reductions of γ-HCH by 84%, 35% and 51% in Isolates 1, 2 and 3 respectively. The three isolates are being reported for the first time as able to degrade γ-HCH. In the future, these microorganisms should be further investigated to establish the pathway and extent to which they degrade γ-HCH. In addition to this, their potential to degrade other persistent organochlorine insecticides and HCH isomers could be evaluated. This will be helpful in determining their potential use as non-polluting bio-agents that rapidly detoxify and/or mineralize recalcitrant and obsolete chlorinated organic pesticides.

CHAPTER 1

1 Introduction

In the quest to ensure better health for himself, his crops and animals man has developed and used synthetic pesticides to combat and exterminate harmful and/or undesirable insect pests. While this has led to remarkable outcomes in public health programs and in the agricultural sector, unexpected adverse environmental outcomes have also been observed (Adeola, 2004). This includes toxicity to non-target species, loss of biodiversity and increase in pest resistance (Tuzimski, 2012). In humans, pesticides have been reported to have disruptive effects on the endocrine, reproductive and immune systems (Kleanthi *et al*., 2008).

The presence of chlorinated pesticide residues in the environment has been a matter of growing international concern. This is because this chlorinated insecticides; (i) are resistant to decomposition or degradation by normal physical or biochemical processes owing to their strong carbon-chloride bond, (ii) have very low solubility in water and (iii) are highly soluble in hydrocarbon-like environment (lipophilicity), such as the lipid and fatty tissue (Kleanthi *et al*., 2008). Consequently they exhibit prolonged toxic effect in the environment to both target and non-target organisms (Adeola, 2004).

γ-HCH is a chlorinated insecticide that is highly saturated with chlorine substituents. As a broad-spectrum insecticide it has been used in many parts of the world for seed treatment, crop protection and for the control of vector-borne diseases (Philips, *et al*., 2006). Since the discovery of its insecticidal properties in the 1940s, it has been used in two main formulations, technical HCH and lindane. Technical HCH is a mixture of different HCH isomers, α-HCH (60-70%), β-HCH (5-12%), γ-HCH (10- 15%), δ-HCH (6-10%), and ε-HCH (3-4%) (Kutz *et al*., 1991). Lindane is the purified γ-isomer (>99% pure) of HCH (Figure 1).

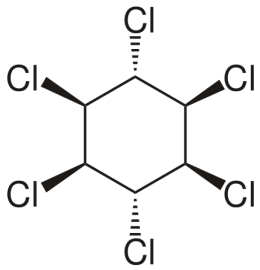

Figure 1: Structure of γ-hexachlorocyclohexane. **(Adapted from ATSDR, 1999).**

In recent years, γ-HCH has become a heavily scrutinized substance owing to its negative environmental effects (persistence in nature, susceptibility to bio-magnification, and toxicity to higher animals) (Kleanthi *et al*., 2008). Consequently, it has been flagged down for regulatory intervention and many countries have either banned or restricted its manufacture, distribution and use. Despite this, large amounts of HCH still remain at the sites where it was produced, used or unsoundly disposed. Sites of HCH contamination and stockpiles of unused technical-HCH and Lindane have been reported in Europe (Concha-Grana*, et al.*, 2006), the Americas (Osterreicher-Cunha*, et al.*, 2003; Philips, *et al*., 2006) and Asia (Prakash *et al*., 2004; Zhu*, et al.*, 2005). In Africa, the 1998 Food and Agriculture Organization (FAO) inventory of obsolete, unwanted, and/or banned pesticides found unused stockpiles of both technical HCH and lindane (2,785 tons of technical HCH, 304 tons of lindane, and 45 tons of unspecified HCH material) scattered in dump sites all around the continent and the Near East (http://www.fao.org).

While various approaches of HCH decontamination exist (chemical treatment, incineration, and use of landfills), they lack widespread application because they are economically restrictive and at times harsh to the environment. Consequently, bioremediation has been proposed as promising and viable avenue of providing in situ detoxification of pesticide-contaminated sites. It is a relatively inexpensive technology that either completely mineralizes a pollutant or transforms it into a biodegradable substance (Phillips *et al*., 2005). In this regards, different organisms have been found that are capable of using chlorinated synthetic compounds as a growth substrate. In particular, several soil microorganisms capable of degrading and utilizing the organochlorine γ-hexachlorocyclohexane as a carbon source have been reported over last two decades (Sahu *et al*., 1990; Adhya *et al*., 1996; Okeke *et al.,*

2002; Nawab *et al*., 2003).

CHAPTER 2

2 Literature review

2.1 Physical and Chemical properties of γ-HCH

The chemical structure and physical properties of γ-HCH affect its solubility, volatility and sorption characteristics; factors that contribute to its transport, persistence and biodegradability. These factors (Table1) are attributed to the orientation of chlorine atoms around the cyclohexane ring and are often used to explain the relative recalcitrance and biodegradability of the compound (Phillips *et al*., 2005).

Table 1: Physical and chemical properties of γ-hexachlorocyclohexane

Property	Value
Boiling point	342 ^{O}C at 760mmHg
Melting point	112 ^{O}C
Vapor pressure	5.30×10^{-3}
Aqueous solubility	17ppm
Air-water partition coefficient (K_{ow})	3.3
Octanol water partition coefficient (K_{oc})	3.0
Organic carbon water partition coefficient	7.8×10^{-6} atm m^3 mol^{-1}

Source: ATSDR (1999).

2.2 Production of γ-HCH

Chemical synthesis of HCH is done by chlorination of benzene under UV light (Walker *et al*., 1999). Theoretically, eight possible HCH stereoisomers (α (two enantiomeric forms), β, *γ*, *δ*, ε, *η*, and *θ*) are formed during this process. Of these, only the stable isomers (α-, β-, γ-, and δ-HCH) predominate in the technical product. Subsequent treatment of the technical HCH with methanol or acetic acid followed by fractional recrystallization concentrates the *γ*-HCH isomer to 99.9% (Willett et al., 1998). γ-HCH is the only isomer with insecticidal properties.

Due to poor record keeping in some countries and proprietary restrictions in others, it is difficult to quantify the global production and usage of γ-HCH (Willett *et al*.,

1998). Despite this, Barrie and co-workers (Barrie *et al*., 1982) estimated that between 1945 and 1992 the production of HCH was 1,400,000 tons, while Volder and Li (1995) estimated that the cumulative, global technical HCH and lindane usage was 550,000 and 720,000 metric tons respectively. The total global consumption of technical HCH was estimated to be as high as 6 000 000 tons (Li *et al*., 1998).

On an isomer basis, Strand and Hov (1996) estimated that the global consumption of α-and γ-HCH between 1960 and 1989 was 403900 and 146700 tons respectively. Presently, the global production of lindane and technical HCH has significantly decreased following its restriction in many countries. Canada and the United States banned technical HCH mixtures in 1971 and 1978. India on the other hand effectively ceased the production of technical HCH in 1997. According to the Pest Control Products Board, http://www.pcpb.or.ke, Kenya banned lindane in the year 2011.

2.3 Fate of γ-HCH in the environment

Once it enters the environment, γ-HCH undergoes different processes (Figure 2). These processes facilitate its transport, partitioning and degradation in the various components of the environment.

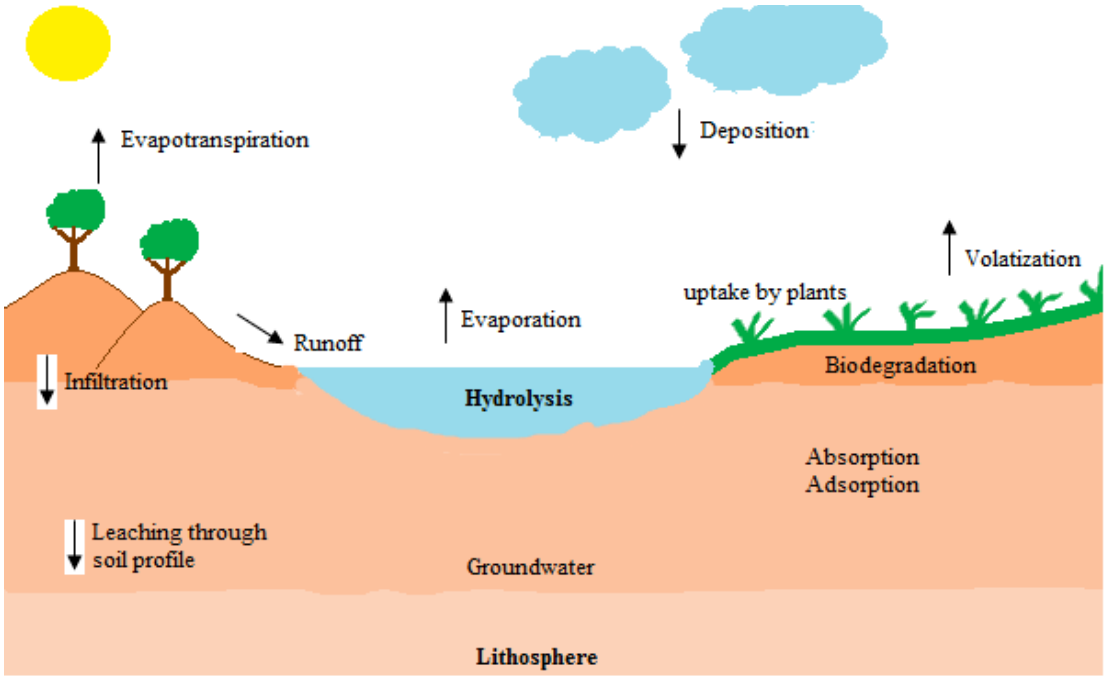

Figure 2: Transport and transformation processes involved in the environmental fate and distribution of γ-hexachlorocyclohexane.
(Adapted from HCN, 1996)

2.3.1 Transport and partitioning of γ-HCH

Volatization: a significant portion of γ-HCH applied for agricultural purposes volatilizes to the atmosphere. In a field study, it was observed that 12 to 30% of the initial amount of γ-HCH applied as canola seed treatment volatilized to the atmosphere (Waite *et al*., 2001). Volatization facilitates the transport of the pesticide by air currents from its site of application to remote locations where it has never been produced or used. Evidence has shown that the global distribution of γ-HCH is facilitated by multiple cycles of evaporation, transport by air and condensation (Wania and Mackay, 1996). Through this phenomenon, the grasshopper effect (Figure 3), γ-HCH evaporates from warmer countries where it continues to be used and travels into the atmosphere towards cooler areas where it condenses out (Li, 1999).

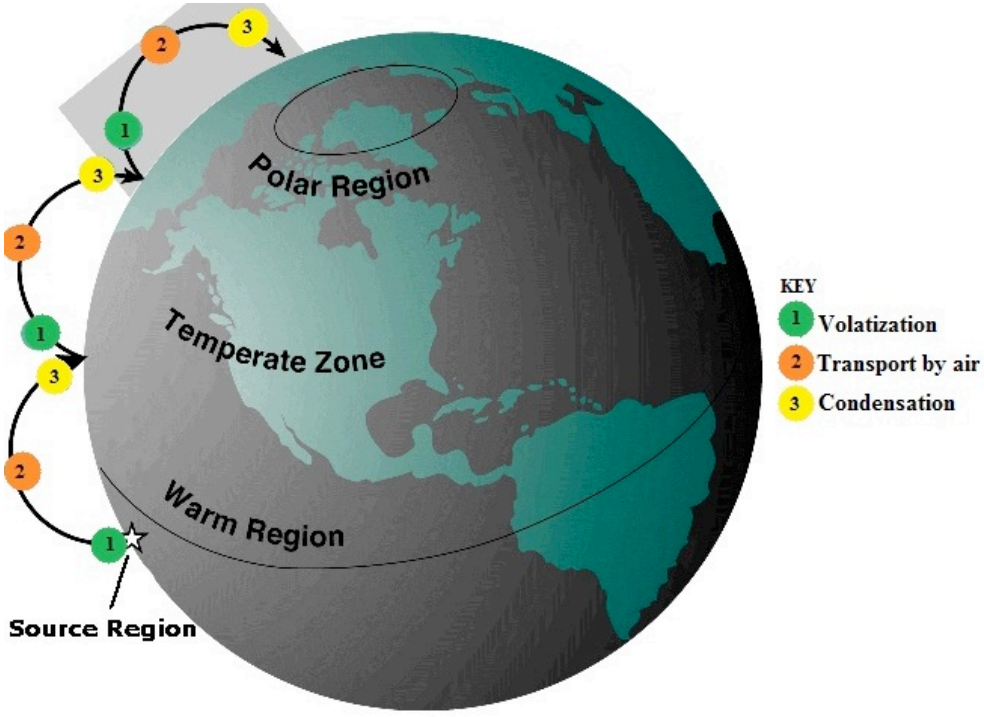

Figure 3: The mechanism of γ-HCH global distribution.(Adapted from http://www.jocara.net/JIOQ/Research/Air_Sampling/POPs_explained.html).

Sorption: Attraction of γ-HCH to charged soil particles and/or organic matter plays an important role in determining its fate and transport. Through columbic attractions between the positively charged γ-HCH and negatively charges soil components (Rathore & Leo, 2012), the bioavailability and mobility of the insecticide is reduced. In a study involving a laboratory sediment/water system (pH=7.42; 2.18% organic

carbon), γ-HCH highly adsorbed on sediments under both aerobic and anaerobic conditions (Wu *et al.,* 1997). In natural water-sediment systems, Saleh *et al.,* (1982) observed that in addition to the organic carbon content of the sediments, diverse natural water-sediment characteristics affect the sorption-desorption behavior of γ-HCH.

2.3.2 Transformation and degradation of γ-HCH

Transformation/degradation constitutes the most important process of HCH elimination. Through these processes, γ-HCH gets transformed to degradation products or is completely mineralized to carbon dioxide. Hydrolysis, photolysis and oxidation are considered the major processes of abiotic pesticide transformation (Rathore & Leo, 2012). For γ-HCH, many studies have observed hydrolysis and photolysis to be the more common processes (ATSDR, 1999).

Hydrolysis: This is the reaction between γ-HCH and water molecules. It involves catalysis by a proton, hydroxide or inorganic ions such as phosphates in the aquatic environment. The hydrolysis of γ-HCH is believed to follow first order kinetics where the rate of its degradation is directly proportional to its concentration (Rathore & Leo, 2012). The hydrolysis of γ-HCH has been observed to be temperature and pH dependent. At pH of 9.3, 7.8 and 7.3, the half-live of γ-HCH was observed as 92 hours, 648 hours and 771 hours respectively (Saleh *et al.,* 1982).

Photolysis: Photo-degradation becomes an important degradation process when there is a high level of UV radiation. Through this process γ-HCH molecules receive energy and get excited, causing them to break up or form less stable bonds that easily break-up later (Rathore & Leo, 2012). Because γ-HCH does not contain chromospheres that absorb light >290 nm, these photolytic processes occur indirectly (Brubaker and Hites, 1998).

In the atmosphere, the photo-degradation of γ-HCH occurs when photo-chemically produced hydroxyl radicals react with γ-HCH. Brubaker and Hites (1998) measured the rate constants for the reaction of γ-HCH with hydroxyl radicals as 1.9×10^{-13} cm^3 molecule/second. In locations where the atmospheric hydroxyl radical concentration

is very low, the persistence times of γ-HCH is much longer (Cortes and Hites, 2000).

In the aquatic systems, the photosensitizing agents involved in the indirect photolysis of γ-HCH include humic and fluvic acids. Malaiyandi *et al.,* (1982) showed that γ-HCH underwent enhanced photolysis when aqueous solutions were spiked with 5 and 25 ppm of soil fluvic acid, and irradiated with natural sunlight. The indirect photolysis half-life of HCH in natural waters has been estimated to be about 270 days (Mill, 1999).

Biotic degradation: The metabolic alteration of pollutants in the environment by microorganisms plays a vital role in determining the overall fates of pollutants. Not only do they contribute to the disappearance of pollutants, but also change their physicochemical properties, thus affecting their transport and distribution behavior among various compartments in the environments. While most forms of living things are able to interact with pollutants and metabolize them to a certain extent, microorganisms are particularly important. This is because unlike most plants and animal, microorganisms' metabolic events occur before pollutants enter the food chain (Matsumura, 1989). Thus, preventing pollutant biomagnification.

2.4 HCH degradation by microorganisms

Isolation by enrichment culture has confirmed the ability of diverse bacterial isolates to degrade γ-HCH both aerobically and anaerobically. In fact, some of these isolates have been shown to utilize HCH as a sole carbon source. Some of the well studied HCH degraders include: several *Pseudomonas* (Tu, 1976; Huntjens *et al.,* 1988; Sahu *et al.,* 1990; Nawab *et al*., 2003) and *Sphingomonas* (Senoo & Wada, 1989; Adhya *et al.,* 1996) species, *Clostridium sphenoides* UQM780 (Heritage & MacRae, 1977), *Clostridium rectum* S-17 (Ohisa & Yamaguchi, 1978), and several other *Clostridium sp*. (Jagnow *et al.,* 1977), a strain of *E. coli* isolated from rat feces (Francis *et al*., 1975), two species of *Bacillus* (Yule *et al.,* 1967), a *Pandoraea sp*. (Okeke *et al*., 2002), *Citrobacter freundii* (Jagnow *et al.,* 1977), and *Rhodanobacter lindaniclasticus* (Thomas *et al.,* 1996).

2.4.1 Aerobic degradation of γ-HCH

The aerobic degradation pathway (Fig. 4) of γ-HCH has been extensively analyzed in the bacterium *Sphingobium japonicum* (formerly *Sphingomonas paucimobilis*) UT26 (Nagata *et al.,* 1999; Endo *et al*., 2005).

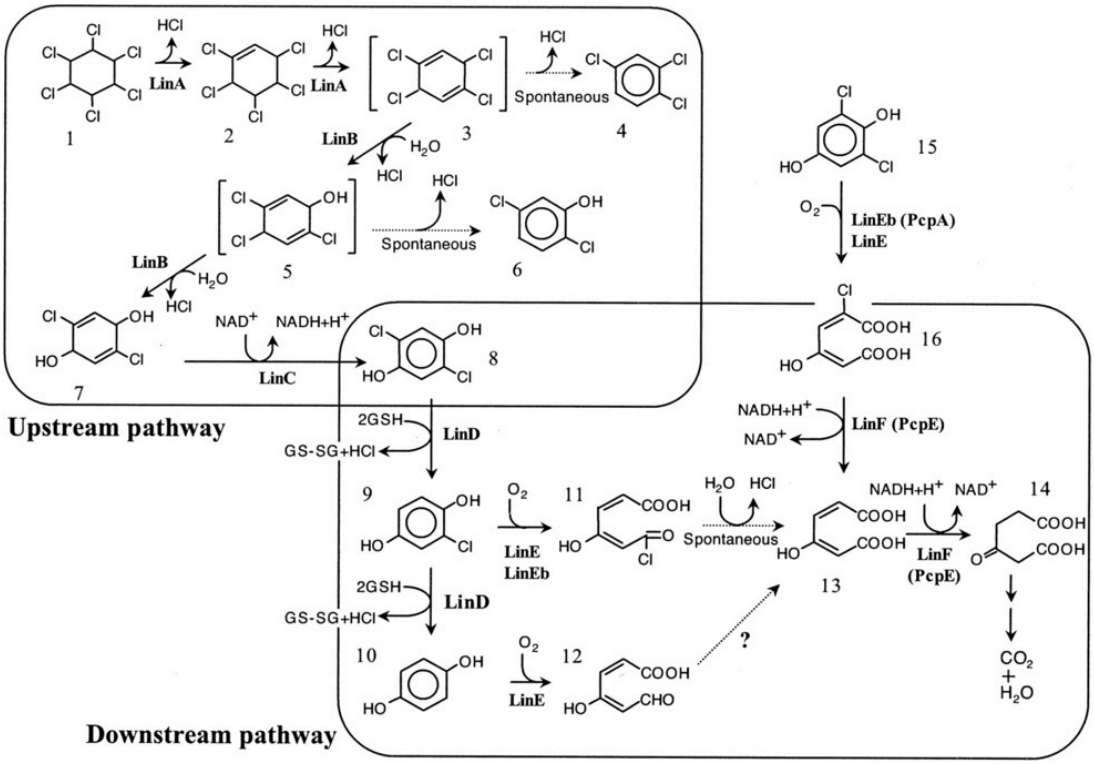

Figure 4: The proposed degradation pathway of γ-HCH in *Sphingomonas paucimobilis* UT26. (Endo *et al*., 2005)

Compounds: 1, γ-hexachlorocyclohexane (γ-HCH); 2, γ-pentachlorocyclohexene; 3, 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 4, 1,2,4-trichlorobenzene; 5, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 6, 2,5-dichlorophenol; 7, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 8, 2,5-dichlorohydroquinone; 9, chlorohydroquinone; 10, hydroquinone; 11, acylchloride; 12, γ-hydroxymuconic semialdehyde (γ-HMSA); 13, maleylacetate; 14, γ-ketoadipate.

In this pathway, two subsequent dehydro-dechlorination steps initiate the metabolic process. *lin*A, via the observed intermediate penta-chlorocyclohexene (γ-PCCH), catalyzes these two reactions to yield the putative product 1,3,4,6-tetrachloro-1, 4-cyclohexadiene (1,4-TCDN) (Imai *et al*., 1991). Subsequently, *lin*B, a hydrolytic dehalogenase, catalyzes two reactions that convert 1,4-TCDN to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) via a second putative metabolite 2,4,5-trichloro-

2,5-cyclohexadiene-1-ol (2,4,5-DNOL) (Nagata *et al.,* 1993). The two putative products of *lin*A and *lin*B catalysis 1,4-TCDN and 2,4,5-DNOL, undergo spontaneous non-enzymatic conversions to form minor dead-end products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol (2,5- DCP) respectively. These minor dead-end products are not degraded by *S. japonicum* UT26 (Nagasawa *et al.,* 1993).

After 2,5-DDOL is converted to 2,5-dichlorohydroquinone (2,5-DCHQ) via a dehydrogenation reaction (Nagata *et al.,* 1994), the enzyme *lin*D reductively dechlorinates 2,5-DCHQ to chlorohydroquinone (CHQ) (*Miyauchi et al,.* 1998). The subsequent break down of chlorohydroquinone proceeds via two routes. In the major route, direct ring cleavage of CHQ results in the formation of an acyl chloride that undergoes spontaneous hydrolytic dechlorination to form maleylacetate. Maleylacetate once reduced to e ketoadipate (Endo et al., 2005), gets converted to 3-oxoadipyl-CoA and acetyl-CoA, both of which are metabolized via the citric acid cycle (Nagata *et al.,* 2007).

In the minor route, chlorohydroquinone is reductively dechlorinated to hydroquinone, which undergoes ring-cleavage to γ-hydroxymuconic semialdehyde.

2.5 Research problem and Justification

The extensive use of γ-HCH over the last six decades resulted in its widespread distribution throughout the global environment. This in turn has resulted in the contamination of aquatic and terrestrial ecosystems, loss of biodiversity and negative health effects to humans and wildlife. Fortunately, some microbes have adapted to the presence of γ-HCH in the environment and have developed the ability to metabolize and/or detoxify it. These microorganisms are useful resources that can be employed in the development of effective γ-HCH bioremediation strategies. Therefore, the purpose of this present work is to isolate and characterize γ-HCH degrading bacteria from organochlorine pesticide contaminated soil in Kenya as potential resources for γ-HCH decontamination.

2.6 Study objectives

Main objective:

To prospect for potential γ-HCH degraders from organochlorine pesticide contaminated soil.

Specific objectives are:

1. To screen and isolate γ-HCH degrading bacteria from contaminated soil.
2. To characterize isolated γ-HCH degrading bacteria according to their biochemical and molecular properties.
3. To examine the biodegradation ability of the isolates.

CHAPTER 3

3 Materials and Methods

3.1 Materials

3.1.1 Reagents, Chemicals and culture Media

The reagents, chemicals and culture media used in the study were all of analytical grade and purchased from standard manufacturers. γ-HCH (99%) was purchased from Sigma, Germany. Oligonucleotides used for PCR were obtained from IDT (Leuven, Belgium).

3.1.2 Soil sample

Organochlorine insecticide contaminated soil was generously donated by Dr Vincent Madadi, (University of Nairobi Pesticides Laboratory, Chemistry department). The soil had been collected from a site in Kitengela (longitude 36^0 East and latitude 1^0 South, approximately 27 kilometers South of Nairobi, the capital city of Kenya) where obsolete and unwanted pesticide stocks had been inappropriately stored. The soils had been stored in a clean aluminum paper at 4 ^{O}C.

3.2 Methods

3.2.1 Screening and isolation of γ-HCH degrading bacteria

Isolation of potential γ-HCH degraders from the contaminated soil sample was done using minimal salt media containing γ-HCH as the sole carbon source. γ-HCH was dissolved in acetone and filter-sterilized to make a stock solution of 0.5 mg ml^{-1}. In a sterilized biological cabinet (Hera Safe KS, Germany), 2ml of the γ-HCH stock solution was added to the bottom of sterilized 100ml volumetric flasks, and the solvent allowed to evaporate. Once all the acetone had evaporated from the flask, 60ml of sterilized M9 minimal salt media (64g Na_2HPO_4, 15g KH_2PO_4, 2.5g NaCl, 5g NH_4Cl, 2ml 1M $MgSO_4$ and 0.1ml 1M $CaCl_2$ per litre pH7) was added to the flasks. To facilitate the detachment of γ-HCH from the bottom of the flask and its distribution through the salt media, the flasks were shaken at 100 revolutions per minute (rpm) for 24 hours. The final concentration of γ-HCH in the media was 16μg ml^{-1}.

Inoculation was done by shaking 10g of the contaminated soil sample in 50 ml of 0.85% sterile NaCl solution and allowing it to stand for 1 hour. The flask was subsequently incubated in a thermoshaker (Gallenkamp, London, England) at 30^{o}C with shaking at 100 rpm. Un-inoculated flasks containing medium and γ-HCH served as a control. After five days of incubation, portions of the culture were spread on LB nutrient agar (10g tryptone, 5g yeast extract 10g NaCl and 15g agar per litre, pH7) and incubated for 48hrs at 30^{o}C. Pure colonies were obtained by serial plating on LB nutrient agar. The pure colonies were used to inoculate fresh preparations of minimal salt media containing γ-HCH as the sole carbon source and monitored for growth.

3.2.1.1 Preparation of glycerol stocks

Glycerol stocks of the bacterial isolates were prepared by mixing 500µl of an overnight culture of the γ-HCH degraders with 500µl 60% sterile glycerol in clean sterile micro centrifuge tubes. The cells were dispersed by brief vortexing and stored at -80 ^{O}C for future use.

3.2.2 Identification of selected bacterial isolates

3.2.2.1 Morphological and physiological characterization

3.2.2.1.1 Gram stain

A smear of pure colony from each bacterial isolate was prepared on clean glass slides and slightly passed over a flame to fix the cells onto the glass. The slides were then flooded with crystal violet, which was allowed to remain on the slide for 60 seconds. The stain was washed off with running tap water. Then, the slides were flooded with gram's iodine for another 60 seconds and the dye washed off using tap water. Decolourization using 95% ethanol and 5% acetone was performed for about 8 seconds, followed by washing and counter-staining with safranin for 60 seconds. The safranin dye was washed off and the slides allowed to dry. The slides were observed under a Leica ICC 50 microscope (Leica microsystems,Wetzlar,Germany) 1000x magnification to determine staining characteristics and cell morphology of the isolates.

3.2.2.1.2 Effect of incubation temperature on Growth

The optimal growth conditions for the individual isolates was investigated using

nutrient broth media (10g tryptone, 5g yeast extract 10g NaCl and 15g agar per litre, pH7). Different flasks containing nutrient broth inoculated with pure cultures of the isolates were incubated at 5, 15, 23, 37 and 45 ^{O}C for 24 hours. The degree of growth was determined by measuring the optical density of the culture using a spectrophotometer at a wavelength of 600 nm. All flasks were prepared in triplicate.

3.2.2.1.3 Effect of pH on Growth

Different flasks containing nutrient broth adjusted to pH 3, 5, 7, 9 and 10 were inoculated with pure cultures of the isolates and incubated at 30^{O}C for 24 hours. Growth was then examined by measuring the optical density of the culture using spectrophotometer readings at 600 nm. All flasks were prepared in triplicate.

3.2.2.2 Molecular characterization based on 16s rDNA gene

3.2.2.2.1 Genomic DNA extraction

Total DNA from the three bacterial isolates was isolated using the alkaline lysis method as described by Kate Wilson (1987). In each case 5ml of an overnight culture was centrifuged (Hettich Zentrifugen,Tuttlingen, Germany) at 5590xg for 5 minutes and the resulting pellet resuspended in 574µl of TE (10mM Tris, 1mM EDTA, pH8) buffer. Into this, 233µl of cell lysis buffer containing lysozyme, SDS, CTAB and proteinase K was added and the mixture incubated for 1 hour at 37 ^{O}C. Subsequently, DNA was separated from the cell lysate using Phenol/Chloroform/Isoamyl alcohol (25:24:1) and Chloroform/Isoamyl alcohol (24:1). Using Isopropyl alcohol, the DNA was precipitated and the pellet resuspended in 50µl of TE buffer. RNase was added to remove RNA before storage at -20 ^{O}C.

The quality of the extracted DNA was analysed by electrophoresis in 1% agarose gel supplemented with ethidium bromide (10 mg/ml). The DNA bands were visualised under a UV transilluminator (Herolab,Wiesloch, Germany).

3.2.2.2.2 PCR amplification of 16S rDNA

PCR amplification of partial 16S rDNA was carried out using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTTACTTGTTACGACTT-3') forward and reverse primers respectively.

Reagents for the reaction were constituted for use as shown in table 2. The thermal cycling parameters were as follows: Initial denaturation for 4 minutes at $95^{O}C$ followed by 35 cycles of amplification ($95^{O}C$ for 1 min, $55^{O}C$ for 1min, $72^{O}C$ for 2 min) and a final extension of $72^{O}C$ for 7 min. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized under UV light after staining with ethidium bromide.

Table 2: Mastermix recipe for 16s rDNA amplification reaction

Reagent	**Concentration**	**Volume (µl)**
PCR grade water		36.5
10x PCR buffer	1x	5
$MgCl_2$ (25mM)	2mM	4
dNTPs (10mM)	200 µM	1
Forward primer	1 µM	1
Reverse primer	1µM	1
DNA template		1
Taq DNA polymerase (5U/µl)	1.25	0.5
Final Volume		**50**

3.2.2.2.3 Sequencing and phylogenetic analysis

The amplified PCR products were purified using QIA quick PCR Purification Kit (QIAGEN, Inc. USA) according to the manufacturer's instruction and sent to the International Livestock Research Institute (ILRI) Kenya, for sequencing.

Using the obtained sequence data, homology searches for the isolates were performed using the BLAST (Basic Local Alignment Search Tool) search program at NCBI (National Centre for Biotechnology Information) obtained from http://www.ncbi.nlm.nih.gov/BLAST. The deduced sequences were then compared to 16s rDNA sequences of related genera from the BLAST search and known Lindane degrading microorganisms in MUSCLE (Edgar, 2004). A phylogenetic tree was then constructed based on these nucleotide sequences with the Bayesian phylogenetic method in MrBayes software obtained at http://mrbayes.net. The trees were then visualised using fig tree software obtained at http://tree.bio.ed.ac.uk/

3.2.3 Biodegradation analysis of γ-HCH by isolates

Biodegradation of γ-HCH by isolate 1, 2 and 3 was evaluated on minimal salt media containing γ-HCH as the sole carbon source. Minimal salt media enriched with γ-HCH was prepared and distributed in sterilized 100ml volumetric flasks as described in section 3.2.1. The flasks were then inoculated with the individual isolates and incubated at 37^{O}C in a rotary shaker at 120 rpm. Growth of the bacterial isolates was monitored on a daily basis by measuring the optical density of the growth media at 600nm. After 10 days of incubation the contents of the flask were extracted for the purpose of quantifying γ-HCH degradation.

The contents of the flask were individually transferred into clean 50ml centrifuge tubes and centrifuged at 16,770xg for 10 minutes. The supernatant was then transferred into clean separating funnels to which 50 ml triple distilled Dichloromethane (DCM) was added. The flasks were vigorously shaken with periodic release of pressure and allowed to stand for 30 minutes. The organic layer was collected into clean round bottomed flasks and the extraction procedure repeated twice. The organic extracts were subsequently dehydrated by passing through anhydrous $Na_2 SO_4$ column. Then 3 ml of iso-octane was added to the extracts as a keeper before concentration to 5ml using a rotary evaporator. The concentrate were collected into clean glass vials and sent to the International Livestock Research Institute (ILRI) Kenya, for GC-MS analysis using the following protocol.

On Agilent GC-MS Workstation U.S.A. instrument, GC was performed using a 7890A GC; the oven temperature was set at 320°C with the program set as 45°C for 1.5 minutes then 20°C/min to 240°C for 2 minutes then 40°C/min to 300°C for 4 minutes. Inlet pressure used was 7.3614 psi at injection temperature 230°C. The carrier gas was helium at a flow rate of 1 ml/min and the run time was 18.75 minutes. The mass spectrum analysis was done using 240MS/4000 electron impact ion source. The mass range was 45–450 *m/z* with a target total ion chromatography of 6000 counts, 25,000 μSecs maximum ion time and 20 μAmps emission current. The run tine was 25 minutes. The obtained spectra were compared with respective internal reference mass spectra of authentic compounds.

CHAPTER 4

4 RESULTS

4.1 Screening and isolation of γ-HCH degrading bacteria

Three morphologically distinct bacterial isolates were able to grow in minimal salt media containing γ-HCH as the sole carbon source. These isolates were selected as potential γ-HCH degraders and designated as Isolate 1, 2 and 3 (figure 5). After 48 hours of incubation on nutrient agar, it was observed that the isolates formed circular convex colonies with entire margins. Isolate 1 had a yellowish color while isolate 2 and 3 appeared off-white.

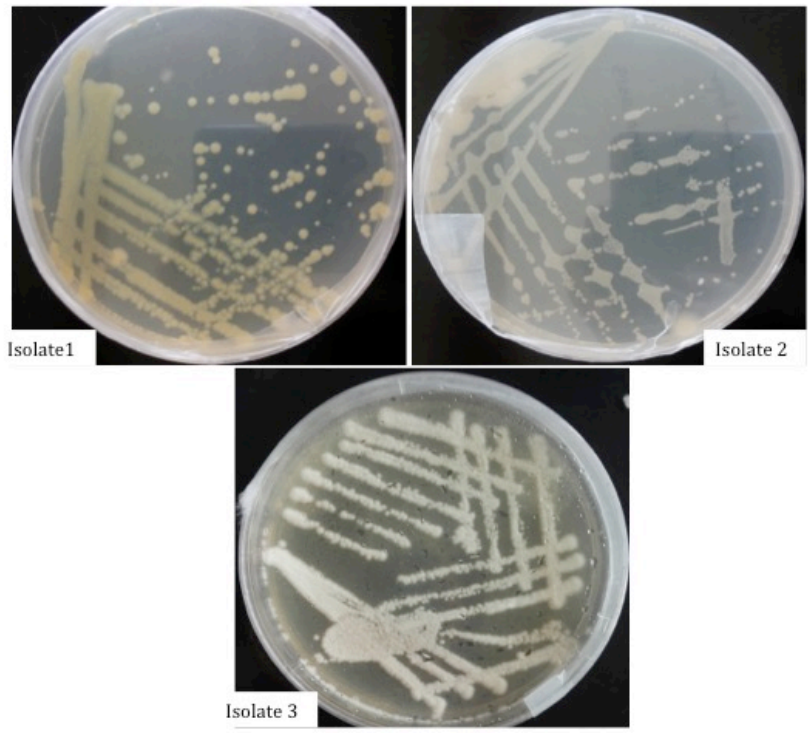

Figure 5: Morphological appearance of bacterial isolates obtained from γ-HCH degrading enrichment culture on nutrient agar.

4.2 Characterization of bacterial isolates

4.2.1 Physical characterization

Gram stain results revealed that the three isolates were all rod-shaped gram-negative bacteria (Figure 6). Their optimal growth temperature and pH were 36^{O}C (Figure 7) and 7 (Figure 8) respectively.

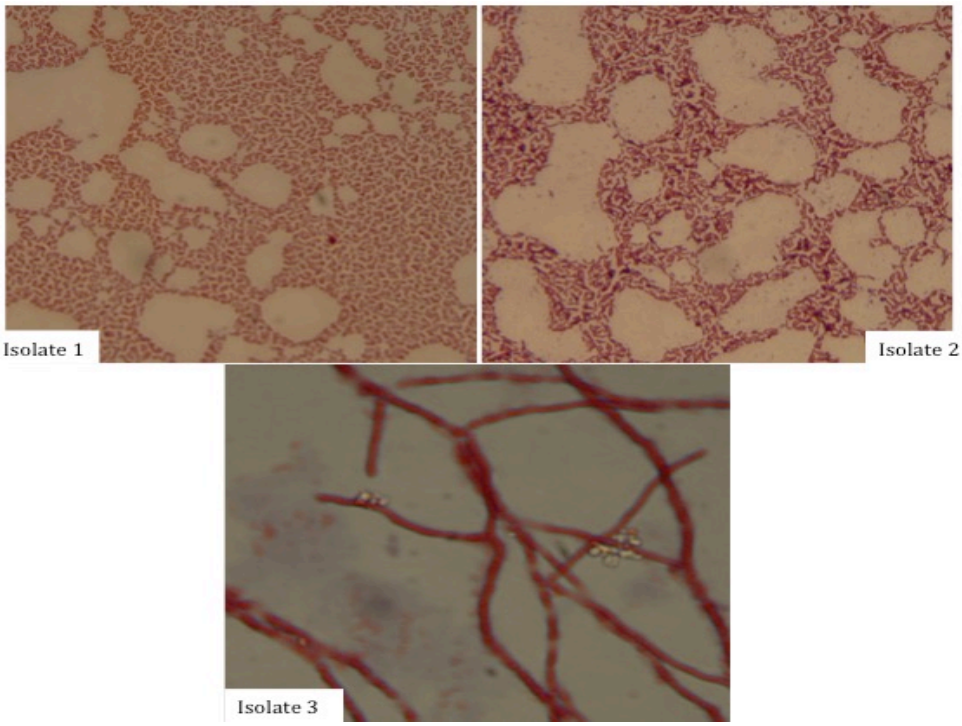

Figure 6: Gram stain characteristics of bacterial isolates under 1000x light magnification.

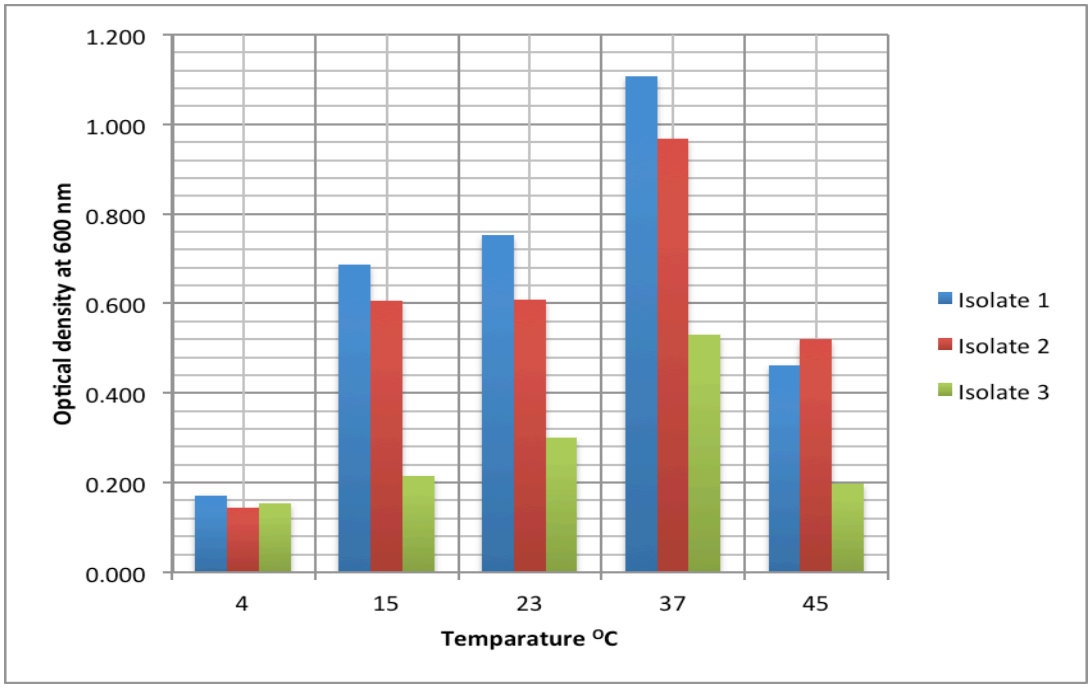

Figure 7: Effect of temparature on the growth of isolated bacteria after 24 hours incubation

Maximum growth was observed at 37 ^{O}C with O.D$_{600nm}$ of 1.107, 0.967 and 0.531 for Isolate 1, 2 and 3 respectively.

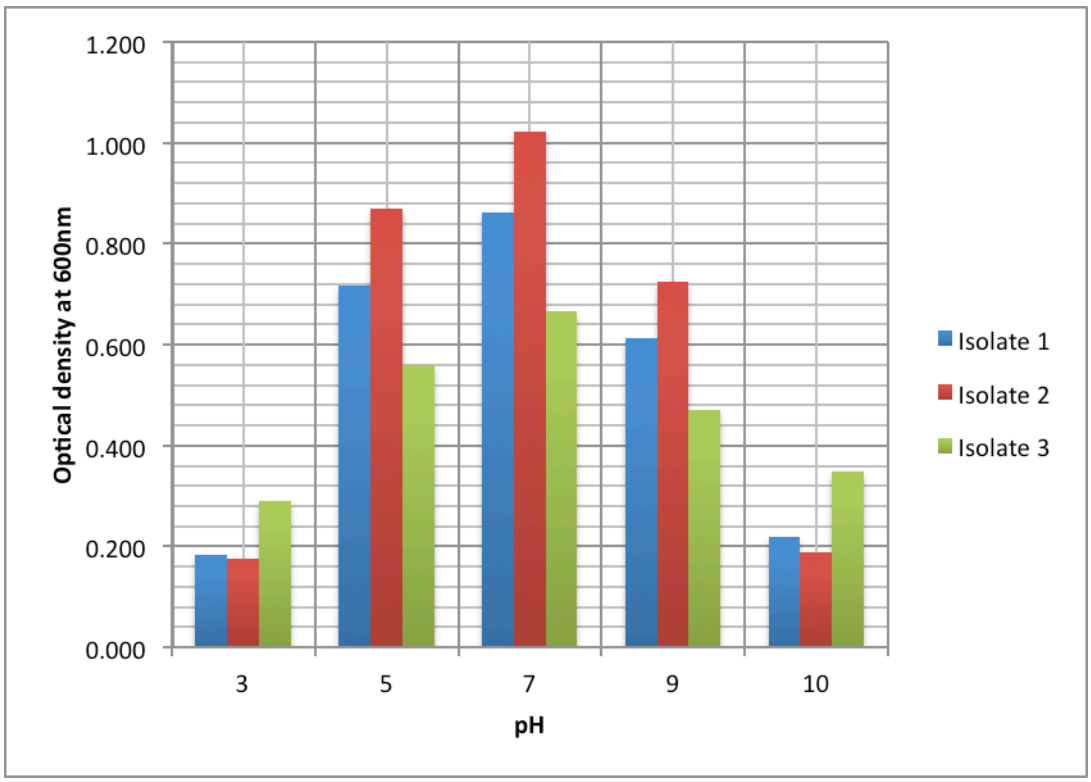

Figure 8: Effect of pH on the growth of isolated bacteria

The optimum growth pH for the three isolates was at 7 with $O.D_{600nm}$ values of 1.107, 0.967 and 0.531 for isolate 1, 2 and 3 respectively.

4.2.2 Molecular characterization

Genomic DNA (Figure 9) extracted from the isolates were used to amplify a partial portion (about 1465bp) of 16s rDNA gene. A blast analysis of the obtained 16s rDNA sequences suggested the affiliation of isolates 1 and 3 to the genus *Achromobacter* and isolate 2 to the genus *Stenotrophomonas*. Isolate 1 and 3 showed maximum identity with *Achromobacter xylosoxidans* IHB (99%) and *Achromobacter xyloxidans* NBRC (97%) respecrively. The query coverage of isolate 1 to *Achromobacter xylosoxidans* was 98% while that of isolate 3 to *Achromobacter xyloxidans* was 98%. Isolate 2 showed maximum similarity with *Stenotrophomonas sp.* SAP52 98% with a query coverage of 98%. The E-values for all the hits was 0. Thus, a phylogenetic tree including the isolates, their selected blast hits and previously described γ-HCH isolates was drawn to examine diversity and interrelationships (Figure 11).

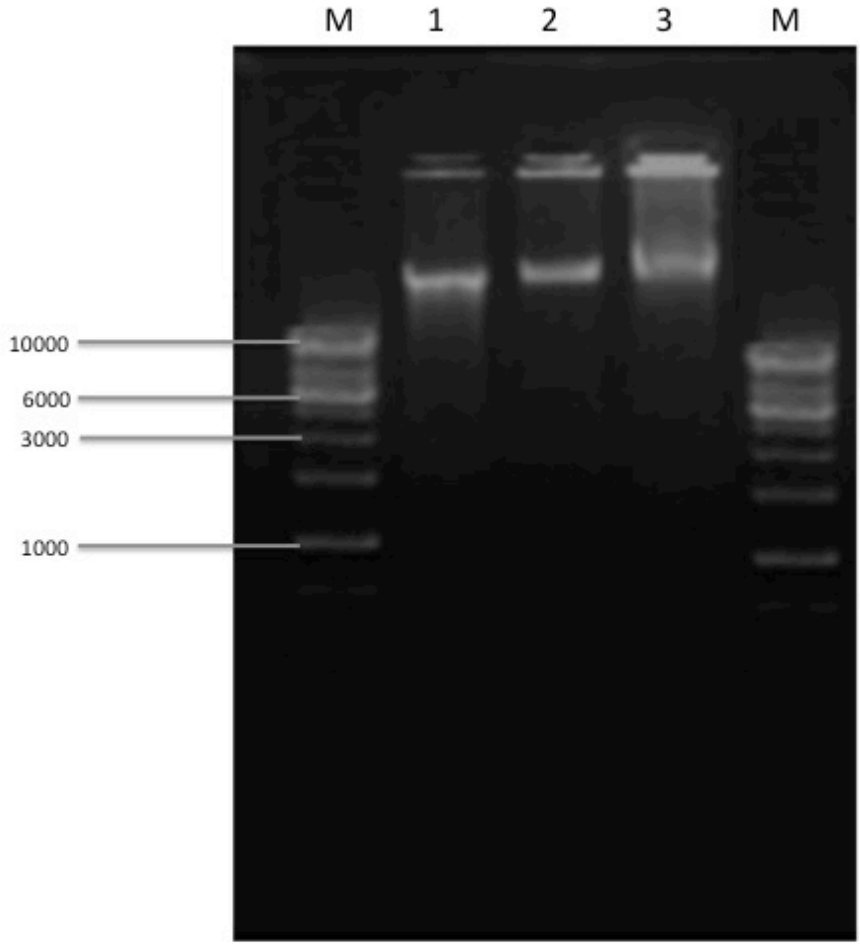

Figure 9: Agarose gel analysis of genomic DNA

Lane M represents DNA molecular weight marker (Roche) and lanes 1,2 and 3 represent genomic DNA from isolates 1, 2and 3 respectively.

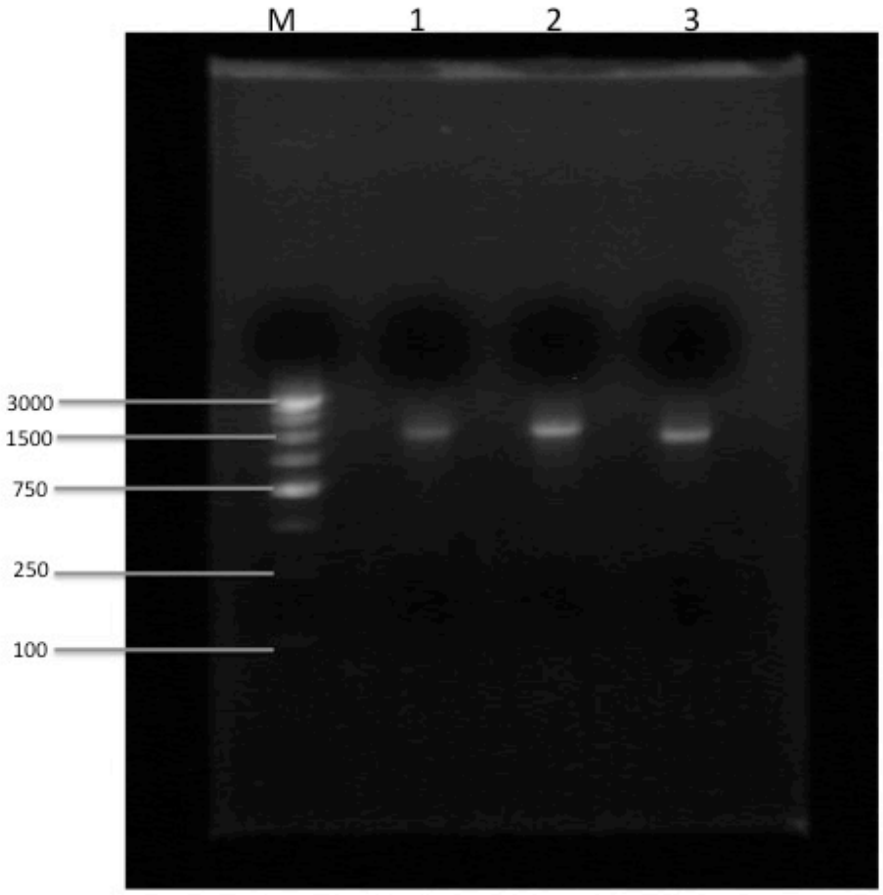

Figure 10: Agarose gel analysis of 16s rDNA PCR amplicons
Lane M represents DNA molecular weight marker (Roche) and lanes 1,2 and 3 represent 16s rDNA PCR amplicons from Isolate 1, 2and 3 respectively. The amplicons lie in line with the 1500 base marker which is within the expected 16s rDNA gene size of 1465 base pairs.

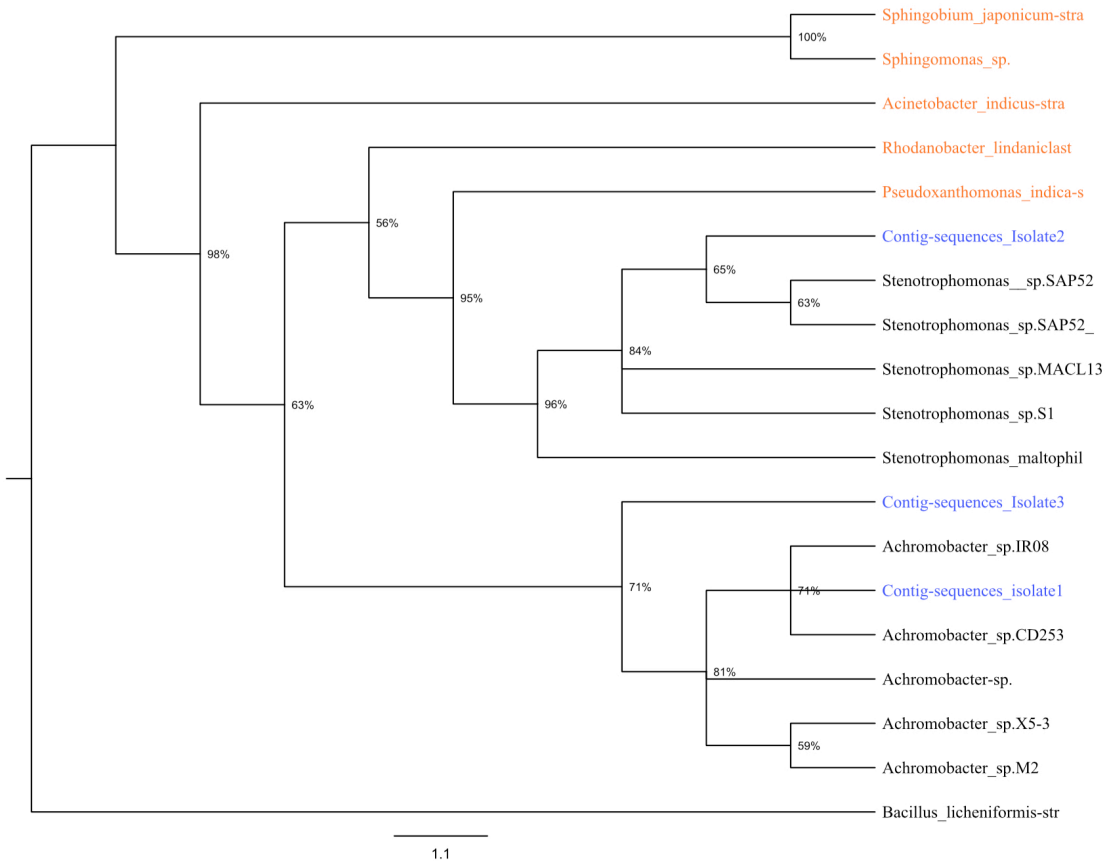

Figure 11: A cladogram based on Bayesian inference of 16S rDNA data from the isolated bacteria.

The figure shows the relationship of the isolated bacterial isolates (blue color) to related bacterial isolates (black color) and Known HCH degrading bacteria (orange color) constructed using 16S rDNA gene sequences. *Bacillus Licheniformis* was used as outgroup. Posterior probability values (in percentage of 1000 replicates) are indicated in the nodes. The scale bar indicates substitutions per site.

4.3 Biodegradation analysis of bacterial isolate

Increase in cell density and disappearance of γ-HCH was used to confirm γ-HCH utilization by the isolates in minimal salt media containing γ-HCH as the sole source of carbon. GC-MS chromatograms of culture extracts showed a decrease in the initial γ-HCH concentration by all three isolates. None of the metabolites observed in the well-studied biodegradation pathway of γ-HCH (Nagata et al., 1999; Endo et al., 2005) were however detected by GC-MS.

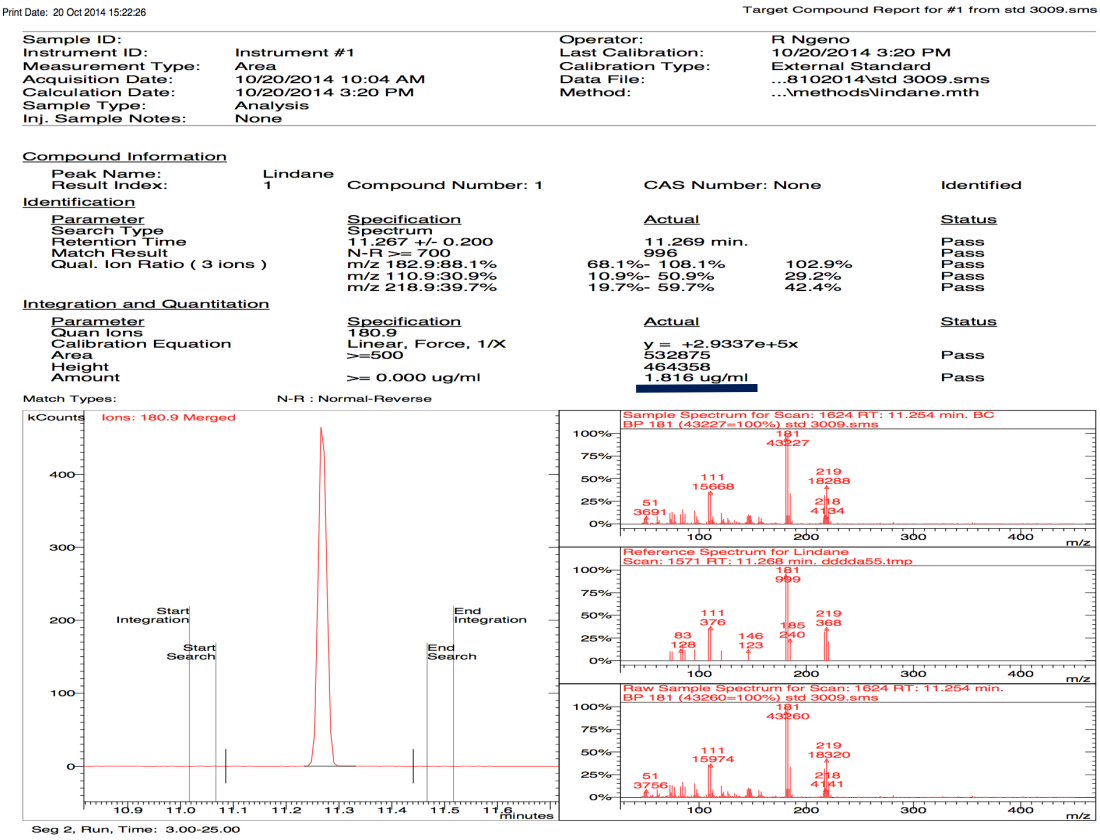

Figure 12: GC-MS chromatogram for extract containing minimal salt media with γ-HCH as sole carbon source before inoculation with bacterial isolates.

The initial concentration of γ-HCH was found to be 1.816 µg/ml.

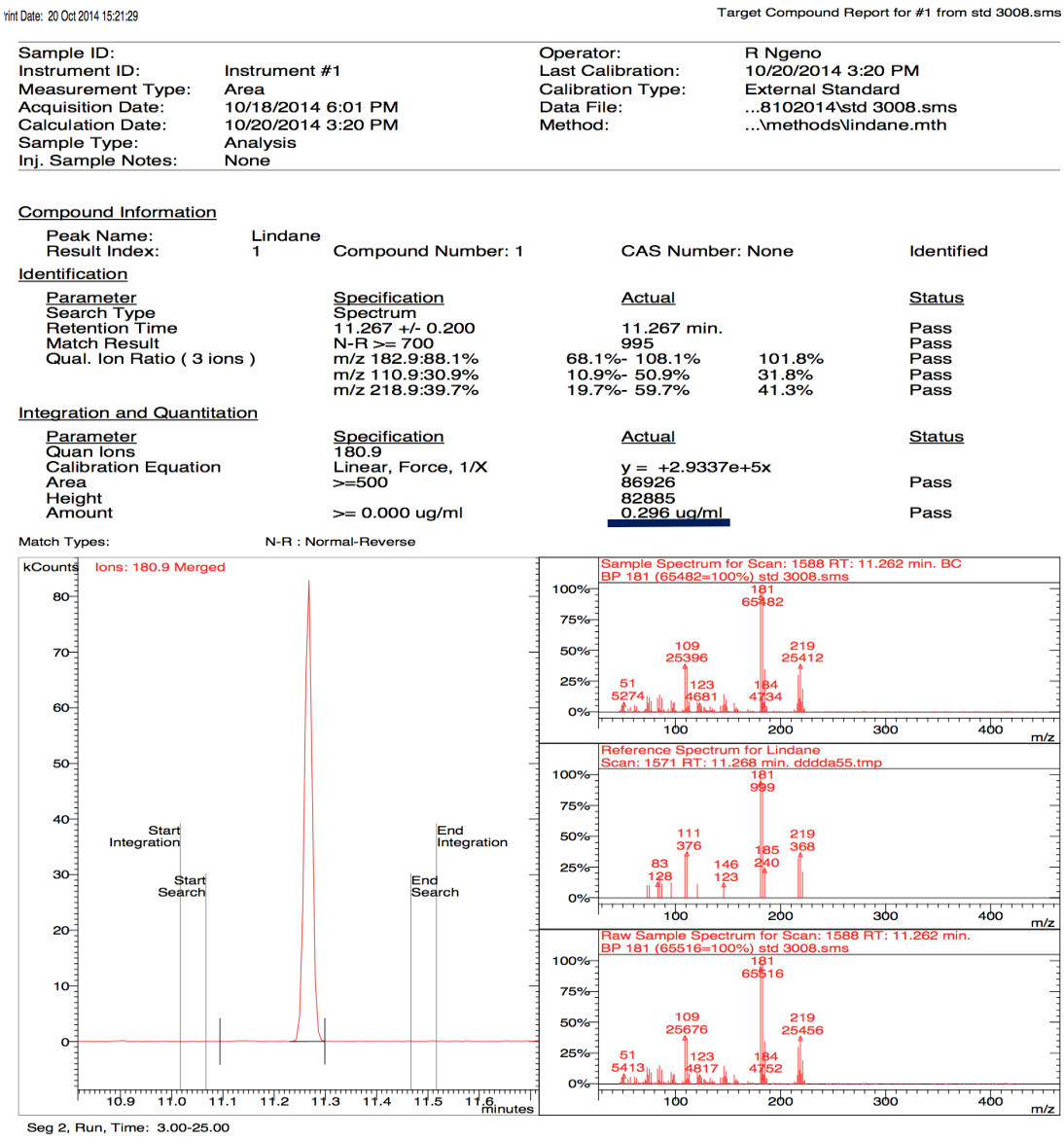

Figure 13: GC-MS chromatogram for extract containing minimal salt media with γ-HCH as sole carbon source after 10 days incubation with isolate 1.

The concentration of γ-HCH is observed to decrease from an initial amount of 1.816 µg/ml to 0.296 µg/ml. This represents an 84% reduction of γ-HCH over a 10-day period.

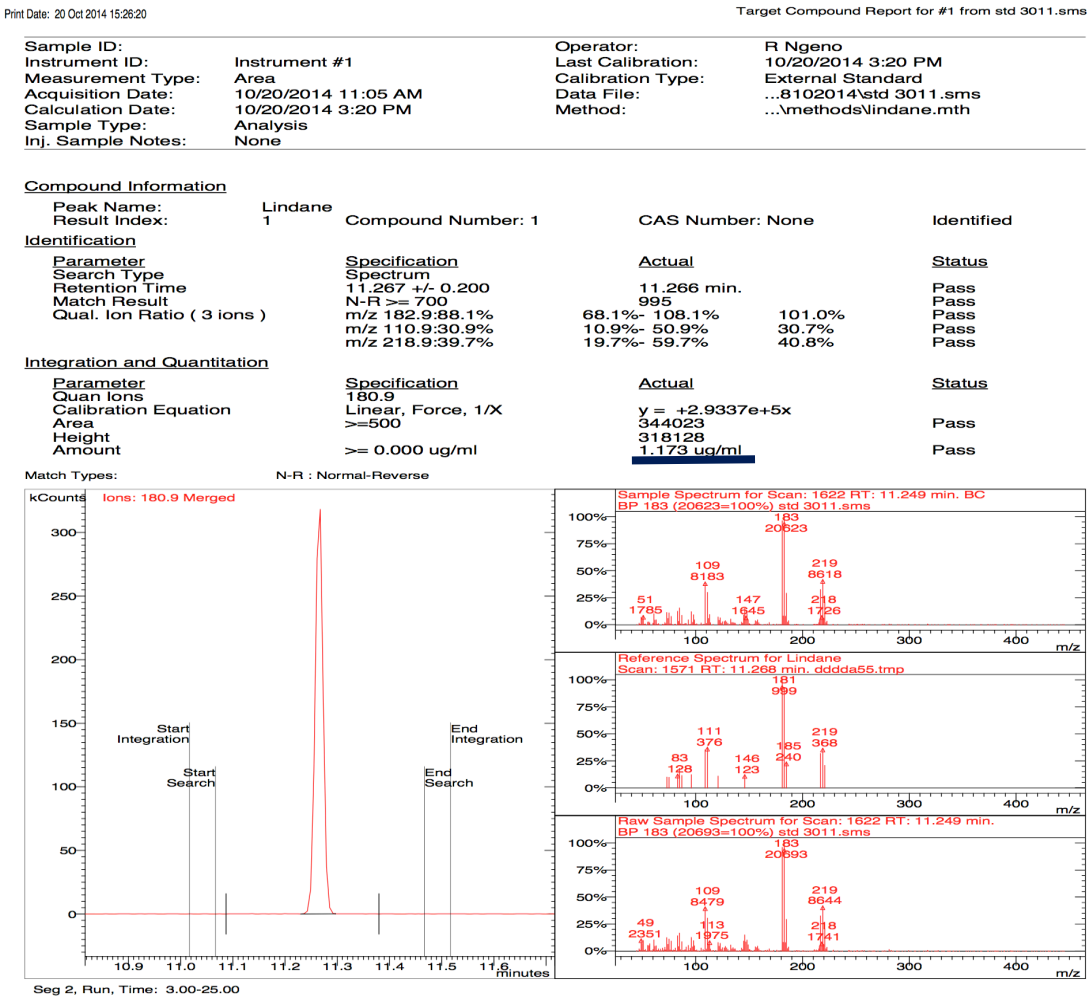

Figure 14: GC-MS chromatogram for extract containing minimal salt media with γ-HCH as sole carbon source after 10 days incubation with isolate 2.

The concentration of γ-HCH is observed to decrease from an initial amount of 1.816 µg/ml to 1.173 µg/ml. This represents a 35% reduction of γ-HCH over a 10-day period.

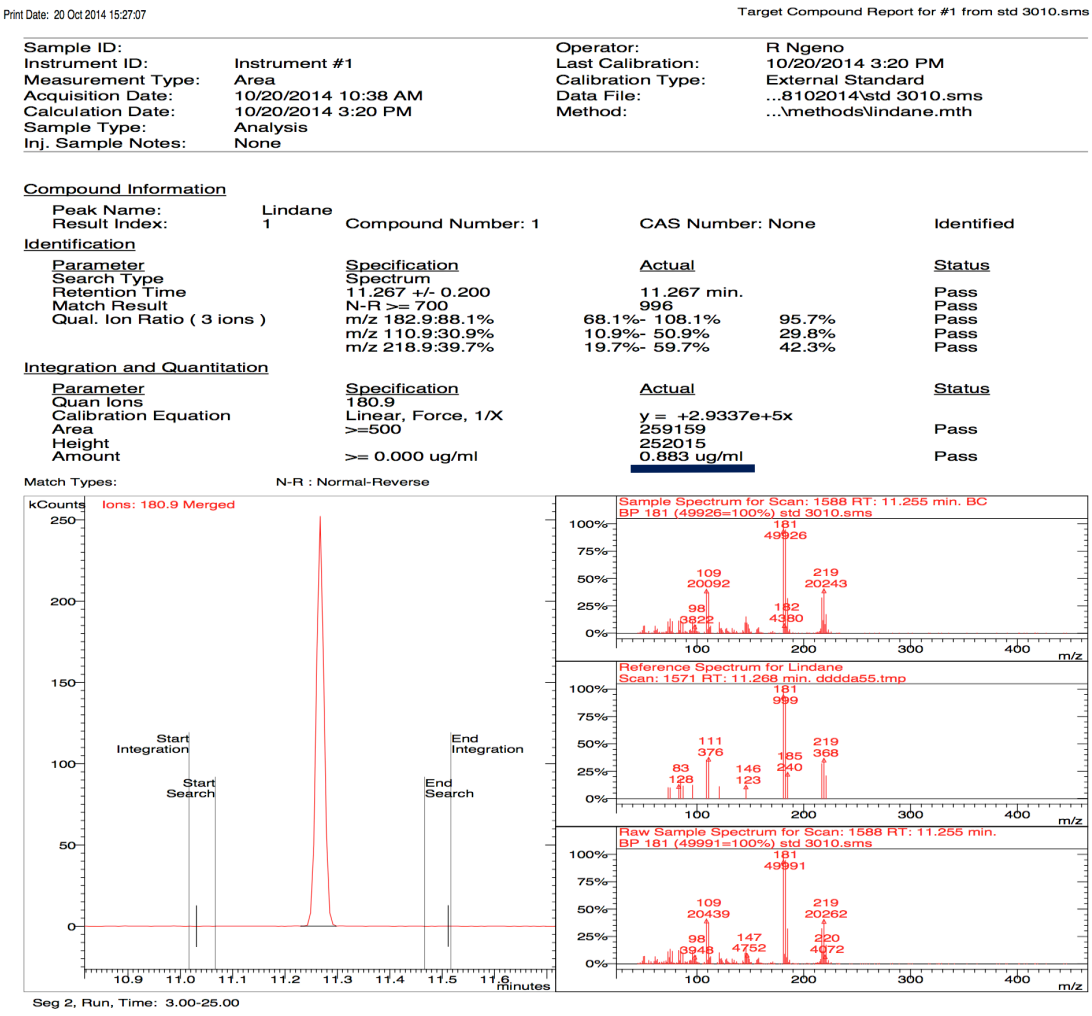

Figure 15: GC-MS chromatogram for extract containing minimal salt media with γ-HCH as sole carbon source after 10 days incubation with isolate 3.

The concentration of γ-HCH is observed to decrease from an initial amount of 1.816 µg/ml to 0.883 µg/ml. This represents a 51% reduction of γ-HCH over a 10-day period

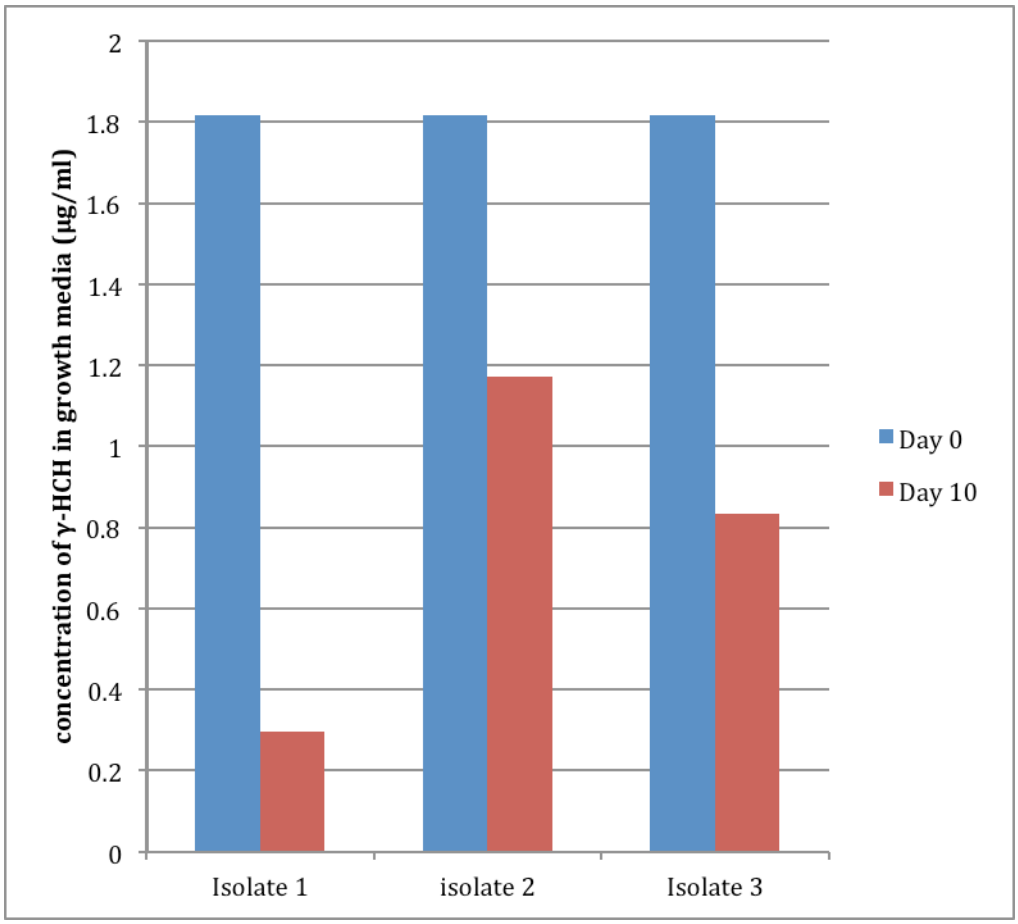

Figure 16: Summary of γ-HCH degradation by isolates after 10 days incubation period.

Isolate 1 observed to have the highest γ-HCH degradation potential followed by Isolate 3 and lastly Isolate 2.

CHAPTER 5

5 Discussion

Chlorinated pesticides have intentionally been introduced into the environment in large quantities as a result of their widespread insecticidal applications. While this has been accompanied by remarkable prosperities in terms of improved agricultural productivity and outstanding control of vector borne diseases, new arrays of unexpected health and environmental problems have developed as evidenced by their negative effects on human health, wild life population and ecosystem diversity.

In light of these deleterious effects, there is an urgent need for the development of safe, efficient and cost effective technologies that guarantee their removal from the environment. In this regards bioremediation has been put forward as a suitable approach. Consequently, there is an increased search of bio-resources capable of biodegrading chlorinated organic pollutants. In the present study, the isolation and characterization of bacteria able to grow in growth media containing γ-HCH as the sole carbon source is reported. Observations made on their morphological characteristics and their phylogenetic relationships are reported and discussed.

5.1 Isolation and characterization of γ-HCH degrading bacteria

In the present work, two *Achromobacter sp*., designated as isolate 1 and isolate 3, and one *Stenotrophononas sp*., designated as isolate 2, were isolated by enrichment method from organochlorine pesticide contaminated soils as able to utilize γ-HCH as a sole carbon source. The isolation of microorganisms capable of degrading specific xenobiotic compounds via culture enrichment often results in the loss of slow or poor degrader. The fact that the three isolates were obtained via this method suggests that they may be robust γ-HCH degraders. In addition to this, the isolates showed good growth behavior over a range of pH (5-9) and temperature (15-37^{O}C). Given that temperature and pH are important factors affecting the ultimate fate of γ-HCH in the environment, the ability of the isolates to grow in different temperature and pH conditions could be an indication of their potential to thrive in different environmental conditions.

The *Achromobacter* and *Stenotrophomonas* genera belong to the phylum proteobacteria. All members of these two groups are aerobic gram-negative rods with their common habitat being water air and soil (Ryan et al., 2009). The fact that the three isolates obtained from contaminated soil sample were all gram-negative rods is in agreement with this body of knowledge.

5.2 Phylogenetic analysis based on 16s rRNA

In the phylogenetic tree presented in figure 11, Isolate 1 and Isolate 3 formed a clade with members from the genus *Achromobacter*, with a posterior probability value of 71%. Within this clade Isolate 1 formed a sister group with *Achromobacter xlosoxidans* Strain IR08 and *Achromobacter xlosoxidans* Strain CD 253. This close relationship suggests that the three share more history in common unlike isolate 3 which appeared to be an out-group of the clade.

On the other hand, Isolate 2 formed a clade with members from the genera *Stenotrophomonas*, with a posterior probability value of 96%. This clade clustered with *Pseudoxanthomonas indica* strain P15, a known γ-HCH degrader isolated from an open HCH dumpsite in India REF??, with a posterior probability value of 95%.

Several workers have previously isolated and identified microbes capable of degrading γ-HCH. The most common of these have been from the genera *Sphingobium, Pseudomonas, Xanthomonas, Devosia* and *Acietobacter* (Philips, *et al*., 2006). In the present work, isolates from the genera *Achromobacter* and *Stenotrophomonas* were observed to grow in minimal salt media containing γ-HCH as the sole carbon source. To our knowledge, members of these two genera have not previously been reported to utilize γ-HCH as a sole carbon source. However, member of these groups have been reported to degrade other xenobiotic pollutants. In degradation studies of endosulfan, another persistent organochlorine pollutant, *Achromobacter xylosoxidans* CS5, originally isolated from activated sludge was found to promote its removal from soil as described by Li *et al*., (2009). Yu *et al.,* (2012) observed that *Stenotrophomonas* sp. LD-6 was able to fully degrade 100 mg/l of endosulfan as the sole source of carbon and sulfur in a period of 10 days. In other studies microorganisms from these two groups have been shown to actively degrade

polycyclic aromatic hydrocarbons (Guo et al., 2008; Boonchan *et al.,* 1998). These findings highlight the important roles played by members of these genera in the removal of a wide range of xenobiotic pollutant and to some extent confirms their potential to degrade γ-HCH.

5.3 Biodegradation studies of γ-HCH by the bacterial isolates

Growth studies (Fig. 13) of the three isolates in minimal salt media containing γ-HCH as the sole carbon source revealed that during an incubation period of 10 days, there was significant increase in cell density that could be correlated to the disappearance of γ-HCH from the culture media. Over a 10-day period, all isolates were able to reduce the initial concentration of γ-HCH by over 30%. This suggests that the isolates were able to derive energy from the substrate to sustain their growth.

During catabolism (where the substrate serves as an energy source), microorganisms have been shown to adopt different processes by which they breakdown and utilize pesticide substrates (Matsumura, 1989). In a non-enzymatic process, (DDT) was photo-chemically degraded by blue green algae through the production of flavoproteins (Essac & Matsumura, 1980). In this process the flavoproteins catalyzed photolysis by acting as photosensitizers that absorbed and transmitted light energy to the pesticide substrate. In enzymatic processes, specialized enzymes catalyze the breakdown of the pesticide substrate to simpler molecules that can be utilized for growth and energy. In the well-studied pathway for γ-HCH degradation in *Sphingomonas paucimobilis* UT26 (Nagata *et al*., 1999), no metabolites were detected in the culture supernatant except for two dead end products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol (2,5- DCP) (Nagasawa *et al*., 1993). These metabolites were absent in GC-MS analysis conducted in the present study. These findings suggest that the isolates could have a γ-HCH degradation pathway that is different from the popular *Sphingomonas paucimobilis* UT26 pathway or that the isolates adopt a non-enzymatic process in their catabolism of γ-HCH. These possibilities provide exciting avenues in the search of alternative γ-HCH degradation mechanisms by diverse organisms from different environmental niches thus improving the current understanding of γ-HCH degradation.

CHAPTER 6

6 Conclusion

From the present study, three bacterial isolates obtained from organochlorine pesticide contaminated soil were able to grow in minimal salt media containing γ-HCH as the sole carbon source were isolated and characterized. From these findings, it can be concluded that environmental samples contaminated with xenobiotic pollutants are a rich source of potential bioremediation microbial resources. Having had exposure to the pollutants, such microorganisms may be well adapted for use in bioremediation interventions as a result of their pre-exposure and acclimatization to the pollutants.

6.1 Future perspectives

The present study mainly focused on the isolation and characterization of bacteria able to grow in media containing γ-HCH as the sole carbon source. To determine the viability of the isolates as effective bio-remediating agents' further work is required. Given that the experiments were conducted in a laboratory setting where all factors affecting the biodegradation of γ-HCH were not taken into account, e.g. presence of alternative carbon source, it would be important follow this work with field trials.

In addition, given that the isolates were obtained from soil contaminated with a mixture of organochlorine pesticides including other HCH isomers, it would be interesting to examine their biodegradation ability on these other equally important environmental pollutants.

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Appendix

Table 3: Concentration of organochlorine insecticide in soil sample

Insecticide	**Concentration (ng/g)**
α-HCH	27
β-HCH	<0.02
γ-HCH	120
Heptachlor	<0.009
Aldrin	1200
Dieldrin	1104400
Endrin	12800
Cis-chlordane	150
Trans-chlordane	190
Mirex	<0.009
o,p'-DDE	360
p,p'-DDE	5200
o,p'-DDD	<0.02
p,p'-DDD	210
o,p'-DDT	2700
p,p'-DDT	10500

Table 4: GenBank accession numbers of organisms used in construction of a phylogenetic tree

GQ409968.1	*Achromobacter xlosoxidans*
DQ887776.1	*Achromobacter xlosoxidans*Strain IR08
DQ361075.1	*Achromobacter xlosoxidans* Isolate X5-3
KJ082093.1	*Achromobacter xlosoxidans* Strain M2
JQ724537.1	*Achromobacter xlosoxidans* Strain CD 253
EF198252.1	*Stenotrophomonas sp* MACL13A
FN796826.1	*Stenotrophomonas maltophilia*
JN872547.1	*Stenotrophomonas sp.* SAP52_1
KC581677.1	*Stenotrophomonas maltophilia* strain IARI-ABL-34
AY179327.2	*Stenotrophomonas sp.* S1
NR_117784.1	*Acinetobacter indicus* strain A648
NR_116019.1	*Pseudoxanthomonas indica* strain P15
L76222.1	*Rhodanobacter lindaniclasticus*
AF039168.1	*Sphingobium japonicum* strain UT26
AY771802.1	*Sphingomonas sp*. Gamma1-7
JQ388689.1	*Bacillus licheniformis* strain RK 202

16s rDNA sequences for isolated bacteria

>Isolate1

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> Isolate 2

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>Isolate 3

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