Characterization of soil bacteria capable of degrading selected organic pesticides applied in horticultural farms in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to my beloved Dad and Mum who despite the loneliness occasioned by my absence, believed in me. To my lovely siblings Dennis and Maureen who encouraged me through to this level of education.

Above all to God, the creator of all beings, who provided strength, health and favor to enable me see this output.

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LIST OF ABBREVIATIONS

EPA	Environmental protection agency
ANOVA	Analysis of variance
HPLC	High pressure liquid chromatography
FAO	Food and agriculture organization
PCR	Polymerase chain reaction
BHI	Brain heart infusion
OPPs	Organophosphorus insecticides
MSM	Minéral salts medium
PPM	Parts per million
MRLs	Maximum residue limits
AChE	Acetylcholine esterase
HCDA	Horticultural crop development authority
OPH	Organophosphorus hydrolase
PCB	Pesticide control board
NCBI	National centre for biotechnology information
BLAST	Basic local alignment search tool
WHO	World health organization

ABSTRACT

Pesticide use has been one of the major factors in improving productivity in agricultural enterprises. Pesticide residues in food and drinking water when ingested affect human health while ecosystems are also affected through loss of biodiversity. The aim of the research was to survey pesticide usage in farms under horticultural production, isolate bacterial strains, evaluate their biodegradation potential of selected pesticides, characterize the bacteria isolates morphologically, biochemically and molecularly. A survey in four horticultural regions of central and rift-valley Kenya showed that out of the twenty two (22) formulations of organophosphorus pesticides used by farmers diazinon had the highest proportion of application at 45.9%., followed by dimekill at 18.9% and least in application was brigade at 5.5%. Linuron was mostly applied with an 18.1% proportion of application followed by isoproturon at 13.2% and the least in proportion of application was Neprapamide at 2.7%. Through enrichment cultures total of thirty one (31) isolates were obtained from diazinon and linuron contaminated soils of which thirteen (13) isolates had the ability to degrade linuron while eighteen (18) isolates could degrade diazinon. Degradation of the pesticides was monitored by High pressure liquid chromatography (HPLC). Bacterial isolates had significant levels ($P<0.05$) of the pesticides degradation. Isolate DJk-4A was the best diazinon degrader at 95.8% degradation followed by isolate DKi-6A at 93.66% while isolate DLoG-8A was the third best degrader at 92.75%. Isolates LJk-5C had the highest level of linuron degradation at 99.36% followed by LWa-2A at 98.94% and the third best degrader was isolate LWa-2C at 95.54%. Identification of various degradation intermediate

metabolites was aided by GC-MS analysis. Diversity of the isolates was assessed through DNA sequencing and BLAST search. Phylogenetic analysis of the 16S rRNA gene sequences showed diazinon degraders clustered into six genera namely; *Paracoccus, staphylococcus, Pseudomonas, Enterobacter, Klebsiella* and *Proteus.* Isolates DJk-4A clustered with *Pseudomonas sp* with a 97% sequence similarity to Pseudomonas *pituda*. Isolate DKi-6B clustered with *Staphylococcus sp* with a sequence similarity of 99% to *Stapylococcus xylosus* while isolate DLoG-8A had a 98-% sequence similarity to *Paracoccus sp.* Linuron degrading isolates clustered into eight genera namely; *Myroides, Lysinibacillus, Arthrobacter, Stenotrophomonas, Burkholderia, Xanthomonas, Pseudomonas* and *Enterobacter*. Isolate LWa-2A was positioned among *Arthrobacter sp* with a 97% identity to *Arthrobacter globiformis.* Isolate LWa-2C clustered with *Burkholderia sp* with a 99% sequence identity with *Burkholderia cepacia*. Isolate LJk-5C clustered with *pseudomonas sp* with a sequence similarity of 98% to *pseudomonas migulae.*

The study showed that isolates from the genera *Pseudomonas, Staphylococcus, Paracoccus, Arthrobacter and Burkholderia* could be used to remediate diazinon and linuron contaminated soils under horticultural production. Further studies should be done to identify and characterize proteins involved in the biodegradation activities of these pesticides.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Horticultural production in Kenya

Agriculture remains the backbone of Kenya's economy. Over 80% of the population is directly engaged in agricultural production with the sector contributing about 24% of the country's GDP and 60% of the export earnings (HCDA, 2008). Kenya's ideal tropical and temperate climatic condition makes it favorable for horticulture production and development. The sub-sector is characterized by a tremendous diversity in terms of farm sizes, variety of produce, and geographical area of production. Farm sizes range from large-scale estates with substantial investments in irrigation and high level use of inputs, hired labour and skilled management to small-scale farms, usually under one acre. Currently the Kenya horticultural industry is the second largest foreign exchange earner after tea (Mehrdad, 2004). However, due to the challenges of pest and diseases the horticulture industry has become one of the major consumers of pesticides (PCB, 2004). Pesticides save up to 40% in crop losses, but misuse or overuse of these chemicals can have considerable environmental and public health consequences (Richardson, 1998). Therefore, improvement in yield is sometimes concomitant with the occurrence and persistence of pesticide residues in soil and water (Ware and Whitacre, 2004).

Pesticides reach the soil through direct application to the soil surface, incorporation in the top few inches of soil, or during application to crops. These pesticides also enter

ground water resources and surface run-off during rainfall, thereby contributing to the risk of environmental contamination (FAO, 2000). Accumulation of pesticide residues in the environment is a worldwide problem and the estimated numbers of contaminated sites have grown significantly to a large number (Cairney, 1993). Biological methods for decontamination promise an improved substitute for ineffective and costly physico-chemical remediation methods, although so far only a fraction of the total microbial diversity (i.e. the culturable fraction with metabolic potential) has been harnessed for this purpose (Debarati *et al.*, 2007).

1.2 Statement of the Problem

Population explosion and diminishing cultivation areas necessitated modernization of agricultural practices to involve the use of both fertilizers and pesticides to maximize on yields. Some of these pesticides have long term effects on the environment thus are a threat to human health, wild life and biochemical cycles. Although bacteria exposed to agrochemicals pollutants can adapt to them by mutating or acquiring degradative genes and proliferating in the environment as a result of the selection pressures created by these pollutants, biodegradation may require extremely long periods of time with the adaptation process and reproducibility being very difficult to predict. Moreover, many waste sites contain mixtures of chemicals with complex chemical structures some of which may interfere with the metabolism of others.

1.3 Justification

The conventional techniques used for remediation are interim solutions since the contamination remains on site, requiring monitoring and maintenance of the isolation barriers long into the future, with all the associated costs and potential liabilities. Biotechnology is highly touted as a potential source of safe, inexpensive, and effective methods for the remediation of sites heavily contaminated with agrochemicals and for the direct treatment of agrochemical wastes (Karns, 1998). Although there have been some notable successes in the use of microorganisms for the degradation of waste chemicals, biotechnology has not yet provided a panacea for farmers or applicators. These difficulties can be surmounted through in-vitro selection of microorganisms or microbial consortia adapted to survival in the unique mix of chemicals present at each site.

Assessing of biodegradation capability of bacterial strains from soils of horticultural farms contaminated with pesticides can be an integral part of bioremediation, since microorganisms can use a variety of xenobiotics compounds including pesticides for their growth, mineralization and detoxification (Kanekar, *et al.*, 2004). Therefore, the low cost and generally benign environmental impact of microbial bioremediation offers an attractive and efficient supplement to more conventional clean-up technologies, which generally have a high public acceptance and can often be easily carried out on sites.

1.4 Objectives

The overall objective was to evaluate the potential use of bacteria in biodegradation of selected pesticide residues in soil under horticultural production.

To survey the use of pesticides in selected horticultural farms

1.4.1 Specific objectives

1. To Isolate and characterize bacterial strains that can degrade the pesticides
2. To quantify the levels of diazinon and linuron pesticides degradation *in vitro*
3. To characterize the diversity of bacteria with capacity to biodegrade linuron and diazinon.

1.5 Hypothesis

1. There is no usage of pesticides in horticultural farms
2. No bacterium exists in soil under horticultural production with pesticide degrading potential.

CHAPTER TWO

2.0 Literature review

2.1 Pesticide use in agriculture

Before the development of synthetic pesticides, farmers used naturally-occurring substances such as arsenic and pyrethrum to control pest and diseases. Widespread use of synthetic pesticides began following World War II (Lewis *et al.,* 1988). The ingredients for many of today's pesticides were in fact, created as weapons of war. Pesticide use was credited with increasing crop yields by reducing natural threats to production and became an integral part of agricultural practices by the mid-1950s. Following World War II, pesticides were a component of what was predicted to be a "green revolution" of abundant food for the world. To date the intensive nature of modern agriculture practices has led to the development and wide spread use of synthetic pesticides in our environment (Bhatnagar, 2001).

Synthetic organophosphorus compounds have been used worldwide as pesticides, petroleum additives and plasticizers (Dragun *et al.,* 1998). These compounds account for more than half of all insecticides used in the world. Statistics have shown that approximately over 5.1 billion ib of organophosphates are applied to agricultural crops annually (EPA, 2001).

Weeds have been a problem in agriculture since about 10,000 BC (Avery, 2006). They have always represented one of the main limiting factors in crop production. Damages

globally caused by weeds are responsible for a loss of 13.2% of agriculture production or about $75.6 billions per year (Oerke *et al.,* 1994). Weeds represent the most important pest complex since they are relatively constant, whereas outbreaks of insects and disease pathogens are sporadic (Gianessi and Sankula, 2006). Apart from the quantitative damages caused by weeds due to competition with water, light nutrients and to the antagonism (parasitism and allelopathy), weeds are able to cause qualitative indirect damages to crop yield reduction and contamination of seeds (Zvonko, 2007). To overcome the problems caused by weeds, herbicides have largely replaced mechanical methods of weed control in agriculture. Herbicides provide a more effective and economical means of weed control than cultivation, hoeing, and hand pulling. Thousands of ureas, also called substituted ureas, have been tested as herbicides and many are in use today (Ware and Whitacre, 2004). They include linuron, diuron and monuron, fenuron-TCA, siduron and tebuthiuron. These herbicides can be used as either selective or non selective weed killer. Their mechanism of action is to inhibit photosynthesis (Donaldson and Kiely, 2002).

2.2 Classification of pesticides

To date about 1000 pesticide formulations or metabolites of pesticides are in use throughout the world (Kamla-raj, 2010). Pesticides include all materials that are used to prevent, destroy and repel pest organisms. Insecticides, herbicides, fungicides and rodenticides are some of the more well-known pesticides (Robert, 1995).

Less well-known pesticides include growth regulators, plant defoliants, surface disinfectants and some swimming pool chemicals. Pesticides are also grouped or classified according to the pests they control, their chemical structure, how/when they work, or their mode of action (Hajslova *et al.*, 1999).

Pesticides with similar structures have similar characteristics and usually have a similar mode of action. Most pesticide active ingredients are either inorganic or organic pesticides (Robert, 1995). Inorganic pesticides do not contain carbon and are usually derived from mineral ores extracted from the earth. Organic pesticides contain carbon in their chemical structure. Most organic compounds are created from various compounds, but a few are extracted from plant material and are called botanicals (Ward *et al* ., 1993). Pesticides can also be classified according to how or when they work. Contact pesticides generally control a pest as a result of direct contact, insects are killed when sprayed directly or when they crawl across surfaces treated with a residual contact insecticide. Weed foliage is killed when enough surface area is covered with a contact herbicide (Vander Hoff and Van Zoonen, 1999). Systemic pesticides are absorbed by pest or plant and move to untreated tissues.

Pesticides can be classified on the basis of how hazardous they are to human beings (WHO, 1990). Insecticides generally are considered the most toxic pesticides to the environment, followed by fungicides and herbicides. However, exceptions exist for certain herbicides which are highly toxic, and are far more hazardous to the environment than are insecticides (Kumar 2010).

2.3 Challenges of pesticide usage

Ecological effects of pesticides are varied and are often inter-related to each other (U.S.EPA, 2002). Water soluble pesticides are easily transported out of the target area into ground water and streams. Fat soluble chemicals are readily absorbed in insects, fish, and other animals, often resulting in extended persistence in food chains (Yadav, 2007). Some of the most troublesome pesticides to the ecology as per WHO classification are (a) insecticides: DDT, dieldrin, diazinon, parathion, and aldicarb; (b) herbicides, 2-4-D, atrazine, paraquat, and glyphosate, and (c) fungicides: benomyl, captan, mercury, copper, and pentachlorophenol.

Organophosphorus compounds have a high index of toxicity and many chronic ecological effects that go often unnoticed by casual observers, yet have great negative consequences in the environment (David *et al.*, 2005). They have similar chemical structures and therefore, similar mechanisms of toxicity of inhibiting acetylcholine breakdown in synapses and red blood cell membranes (Lotti, 2002). These pesticides are highly toxic to target and other non-target animals (Galloway and Handy, 2003). Approximately 3 million poisonings and 300,000 human deaths occur per year owing to organophosphorus ingestion (Bird *et al.,* 2008). Organophosphate pesticides poisoning resulting in death have also been reported from Kenya (Kimani and Mwanthi, 1995). Acute or chronic toxicity of organophosphorus compounds has also been implicated in a range of nerve and muscular disorders (Ragnarsdottir, 2000).

Linuron [3-(3,4-dichlorophenyl)-1- methoxy-1-methyl urea] is a selective N-methoxy-N-methyl is a substituted phenylurea herbicide that is considered to be moderately to

highly toxic to aquatic organisms (Orme and Kegley, 2006). The herbicide is also moderately persistent and relatively immobile in soil, runoff and leaching can result in the migration of the compound to surface- and groundwater bodies, it has frequently been detected in surface and ground waters near or below areas with intensive use, and in one extreme case, linuron was detected in a drinking-water well in concentrations up to 2,800 µg liter^{-1} (Caux *et al.*, 1998). The herbicide is suspected to act as an androgen receptor antagonist affecting the male reproductive system, and an endocrine disruptor (McIntyreet *et al.*, 2002).

2.4 Bioremediation of environmental pollutants

Bioremediation is a process which utilizes the microbial ability to degrade and/or detoxify chemical substances on sites contaminated with pesticides and other recalcitrant compounds. *In situ* bioremediation exploits the ability of microorganisms to reduce the concentration and/or toxicity of a large number of pollutants on site (watanabe *et al.*, 2001). Bioremediation is an economical, versatile, environment-friendly and efficient treatment strategy, and a rapidly developing field of environmental restoration (Rakesh *et al.*, 2005). A well-known example of bioremediation which highlighted the usefulness of this treatment strategy and accelerated its development was the biological clean up of a large accidental oil spill by the tanker *Exxon Valdez* which ran aground on Bligh reef in the Gulf of Alaska in March 1989, spilling approximately 41,000 m^3 of crude oil and contaminating about 2000 km of sea shore (Margesin and Schinner, 1999).

Modern scientists, have created pesticides with chemical structures not found in nature, these unique structures are often responsible for a pesticide's effectiveness and also explain why pesticides can persist in the environment (Muller and Korte, 1976). A pesticide's environmental persistence largely depends on its chemical structure and the presence of unusual functional groups, which are large sub-structures within the pesticide molecule. The chemical structure helps determine its water solubility and consequently, its bioavailability, since microbes more readily assimilate water-soluble compounds (Buyuksonmez *et al.*, 2000). Microorganisms have developed many enzymes that can break down natural compounds example, esterase and phosphatase (Lemmon and Pylypiw, 1992). Two other classes of enzymes, mono- and di-oxygenases, are also commonly associated with pesticide degradation by introducing one or two oxygen atoms, respectively, into the structure of a pesticide. This oxidation process often makes the pesticide more amenable to further degradation by increasing its water solubility, thereby increasing its bioavailability to the bacteria (Garcinuno *et al.,* 2006).

2.5 Degradation of organophosphorus compounds

Most organophosphorus compounds have a short half-life in the environment, as they are degraded by microorganisms. In general, organophosphorus compounds do not adversely affect bacteria, because bacteria do not possess Acetylcholine esterase AChE, (Singh and Walker, 2006). Enhanced biodegradation of organophosphorus compounds is also influenced by soil properties and the chemical structure of the organophosphorus compounds. Alkaline soils have been shown to be conducive to a higher degradation

level of OP insecticides (Singh *et al*., 2005). Organophosphorus compounds share similar chemical structures, and therefore soil that developed enhanced degradation for one organophosphorus compound also rapidly degraded other organophosphorus compounds, in a well-known phenomenon called cross-enhanced degradation (Singh *et al*., 2005).

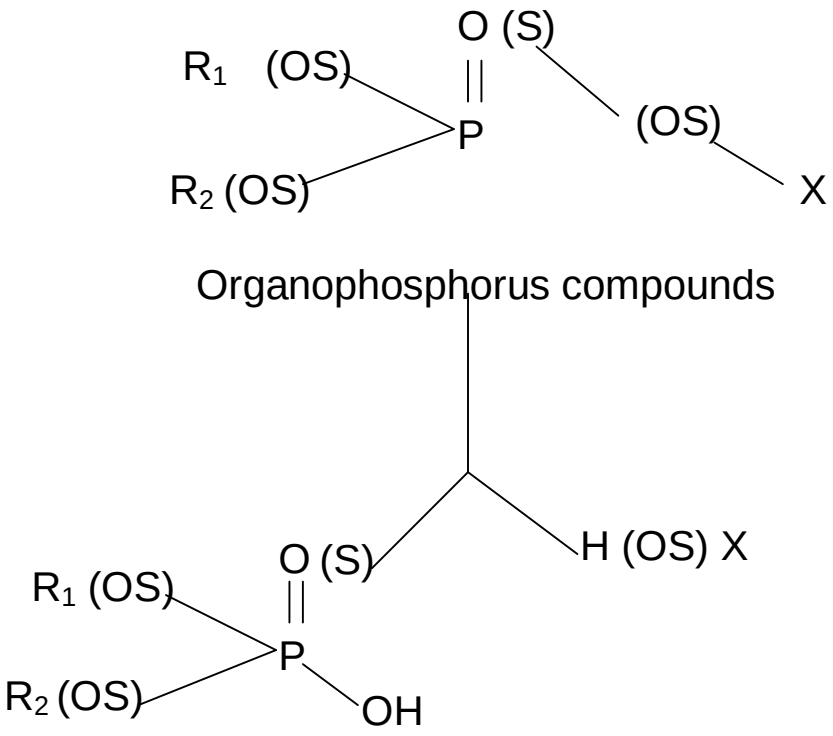

Figure 1: General formula of organophosphorus compounds and major pathway of degradation Sogorb and Vilanova, 2002.

Most organophosphorus compounds are ester or thiol derivatives of phosphoric, phosphonic or phosphoramidic acid. Their general formula is presented in (Figure 1) where 1. R_1 and R_2 are mainly the aryl or alkyl group, which can be directly attached to

a phosphorus atom (phosphinates) or via oxygen (phosphates) or a sulphur atom (phosphothioates). In some cases, R_1 is directly bonded with phosphorus and R_2 with an oxygen or sulfur atom phosphonates or thion phosphonates. One of these two groups is attached with un-, mono- or di-substituted amino groups in phosphoramidates. The X group can be diverse and may belong to a wide range of aliphatic, aromatic or heterocyclic groups. The X group is also known as a leaving group because on hydrolysis of the ester bond it is released from phosphorus (Sogorb and Vilanova, 2002).

2.6 Degradation of diazinon

Diazinon released to surface waters or soil is subject to volatilization, photolysis and hydrolysis. It has been shown to volatilize from both water and soil media and decomposes above 120 °C (HSDB, 2006). Diazinon is also degraded in the environment by hydrolysis. Measured levels of hydrolysis were fastest under acidic conditions (t1/2 = 12 days at pH 5). Diazinon has a relatively short half-life in water, ranging from 70 h to 12 weeks depending on pH, temperature, and sunlight as well as the presence of microorganisms (Scheunert *et al.*, 1993). Isopropyl methyl hydroxyl pyrimidine (diazoxon) is a toxic primary degradates of diazinon hydrolysis (Figure 2) however, diazoxon is rapidly hydrolyzed to oxypyrimidine (IMHP) which is less toxic and more mobile in the environment compared to the parent compound (U.S Environmental Protection Agency, 2000). Diazinon is also broken down by photolysis yielding into diazoxon, a toxic metabolite, and 2-isopropyl-6-methyl-4-hydroxypyimidine (IMHP or oxypyrimidine), a persistent, less toxic product (Bavcon *et al*., 2003).

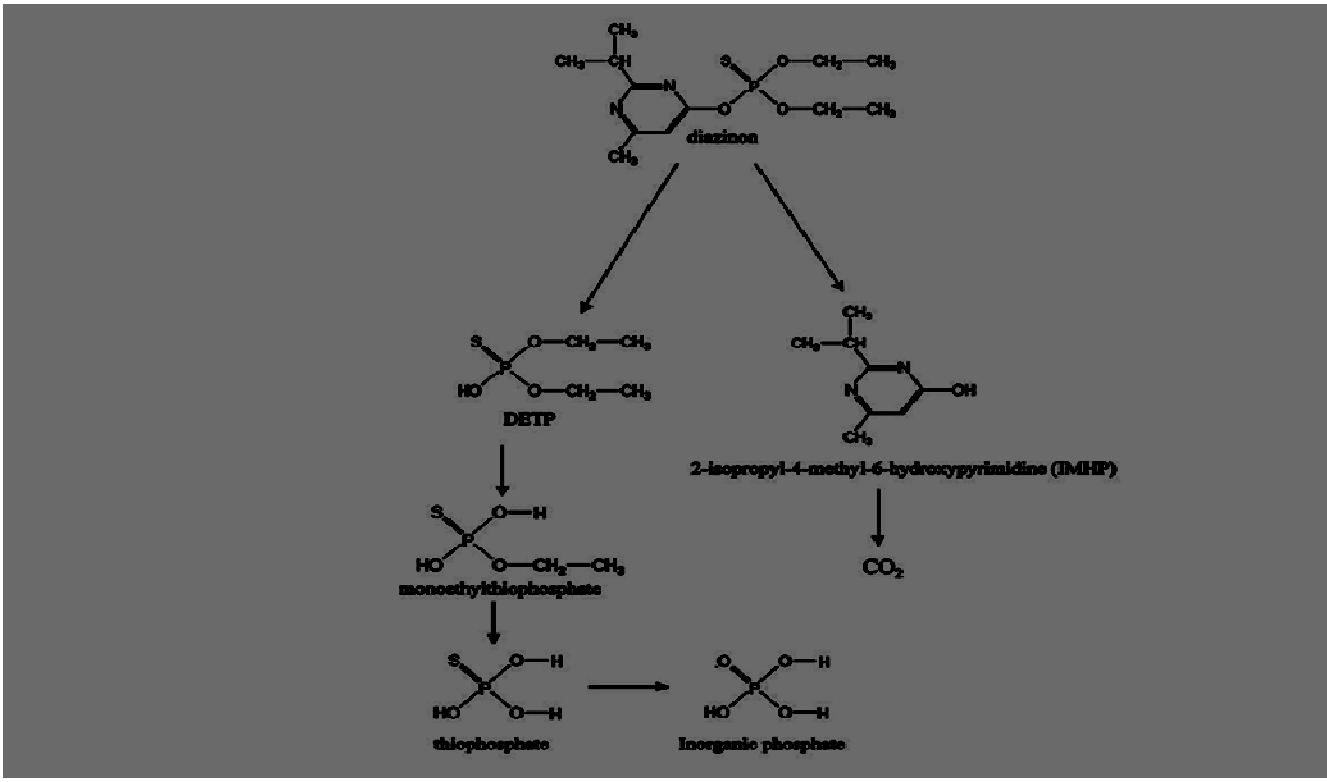

Figure 2: Metabolic pathway of diazinon degradation by soil bacteria Ohshiro *et al.,* 1996.

2.7 Degradation of phenyl urea compounds

Phenyl urea herbicides contain a single chlorinated phenyl ring with a substituted urea moiety. These compounds are high production volume chemicals produced at a level of > 1 million pounds per year and they are widely distributed in the environment due to their low biodegradability (Guzella *et al.,* 2006). All of these compounds have estimated half- lives exceeding 100 days in freshwater and have the potential to bioaccumulation in freshwater organisms (Ahtiainen *et al.,* 2003). Bacteria are able to transform most of these herbicides to mono- and dichloroanilines compounds which are their main

intermediate metabolites (Miller *et al,*. 2010). The fate of these chloroanilines metabolites is not certain, but they may undergo slow degradation, bioaccumulate or attach to particles. Herbicides are hydrolyzed at the urea bridge (Figure 3) resulting in chloroanilines which are degraded by deamination and hydroxylation producing chlorocatechols. Ortho- or meta cleavage pathways oxidize chlorocatechols to 3-chloro-cis,cis-muconate or 5-chloro-2-hydroxymuconic acid semialdehyde, respectively (Di Corcia *et al.,* 1999). These products are then degraded by intermediary metabolic pathways resulting in carbon and nitrogen assimilation.

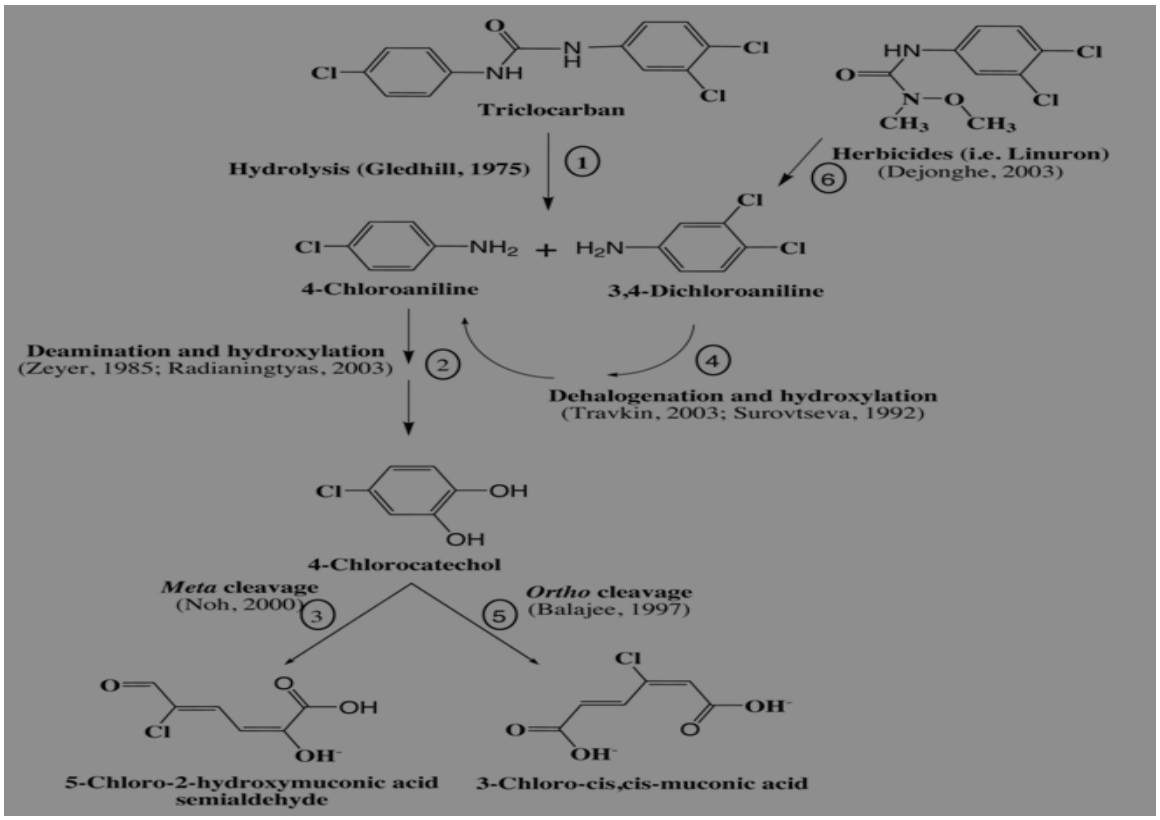

Figure 3: Pathway for the bacterial degradation of phenylurea herbicides Dejonghe *et al.*, 2003.

2.8 Biodegradation of linuron

The levels of dissipation in agricultural soils determined by laboratory and field experiments are highly variable, with values ranging from days to several years (Torstensson and Stenstro, 1990). Under field conditions, the half-life of linuron varies from 30 to 150 days for different soil types, with an estimated average half-life of 60 days (U.S. EPA, 2000). Although (photo) chemical and physical processes may be involved in the removal of this compound, microbial biodegradation is reported to be the most significant mechanism for its dissipation from soil. A consortium of microbes rapidly mineralized linuron and demonstrated a stable degradation activity upon subculture however; isolation of the key organisms seemed to be difficult (El-Fantroussi *et al*., 2000). Only (Cullington *et al.,* 2001) isolated *Bacillus sphaericus* ATCC 12123 and *Arthrobacter globiformis* D47, which can use the alkyl chain of linuron as the sole N and C source. However, these pure strains can only partially degrade linuron, since 3,4-dichloroaniline (3,4-DCA), one of the main potential metabolites in the degradation pathway of linuron accumulated in the medium (Tixier *et al.,* 2001). One reason for incomplete microbial degradation is the presence of a variety of substituted groups on the aromatic backbone, each of which requires slightly different catabolic enzymes for total breakdown. The collection of these enzymes would more likely be present in consortia rather than in single bacteria (Sighn and Walker 2006).

However mineralization of linuron including the mineralization of 3, 4-DCA and N, O-DMHA has recently been reported, by Dejonghe *et, al*., 2003 and Sorensen *et al.*, 2005. Both studies showed contrasting findings Dejonghe *et al.*, 2003 showed that a synergistic consortium was involved in complete linuron mineralization. In the consortium, *Variovorax sp*. WDL1 was the primary linuron degrader and responsible for the transformation of linuron into 3, 4-DCA and N O-DMHA based on pure cultures. However, although the strain was able to also metabolize 3, 4-DCA, transient accumulation of 3, 4-DCA resulted apparently in ineffective linuron degradation. Efficient linuron degradation by *Variovorax sp*. WDL1 in the consortium was shown to rely on coexisting members, i.e. the 3,4-DCA degraders *Delftia acidovorans* WDL34 and *Comamonas testosteroni* WDL7, and the NO-DMHA degrader *Hyphomicrobium sulfonivorans* WDL6 (Figure 4). In contrast, Sorensen *et al.*, 2005 concluded that a single organism, *Variovorax* sp. SRS16, mineralized linuron independently of other bacteria. This strain is closely related to *Variovorax* sp.WDL1, and it was suggested that only members of this genus that are able to mineralize linuron.

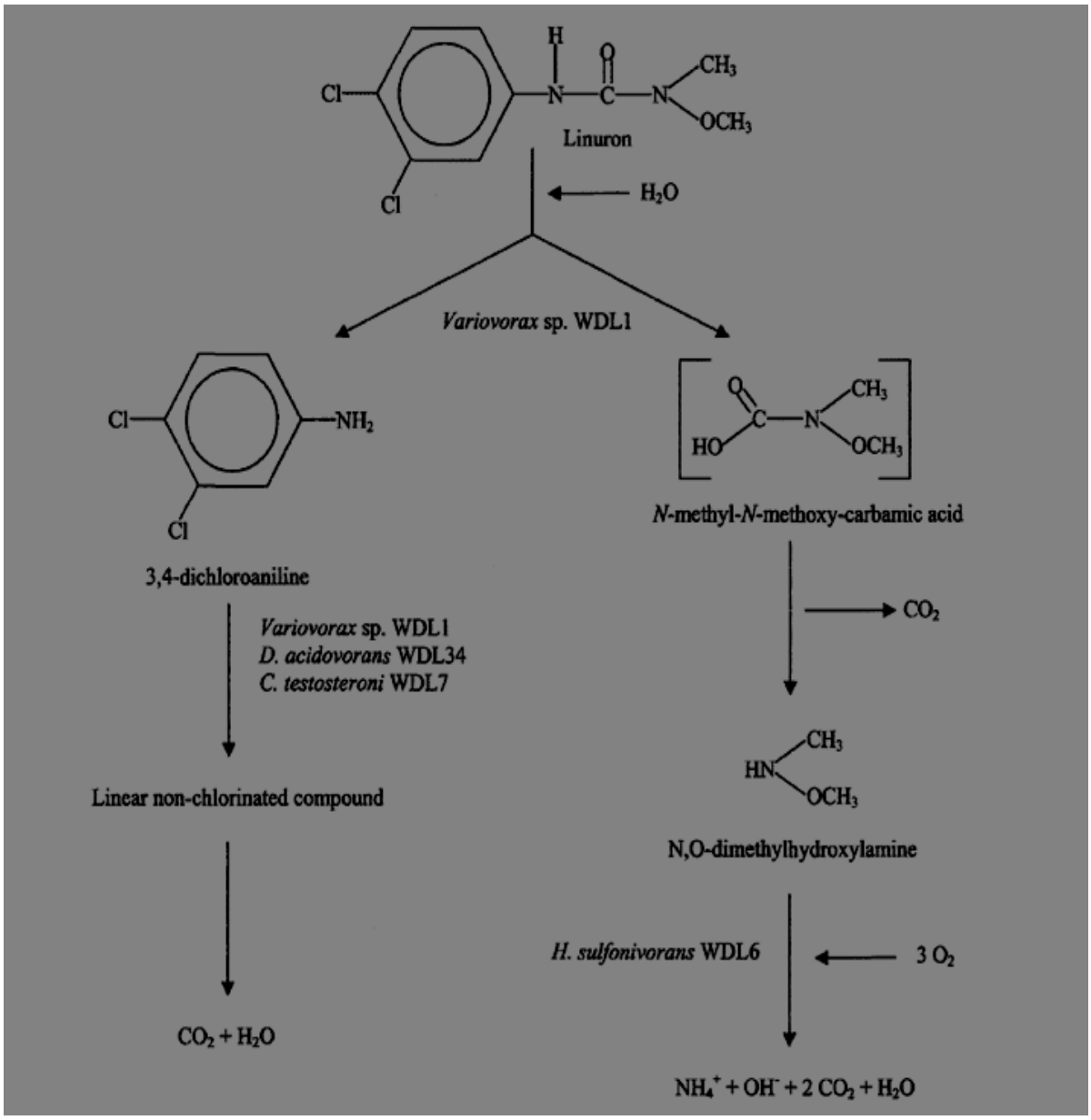

Figure 4: Degradation pathway of linuron by bacteria (Dejonghe et al., 2003).

2.9 Enzymatic degradation of diazinon and linuron

Diazinon like other Organophosphorus compounds has phosphorus usually present either as a phosphate ester or as a phosphonate. Being esters they have many sites which are vulnerable to hydrolysis. The principal reactions involved are hydrolysis, oxidation, alkylation and dealkylation (Singh *et al.*, 2005). Microbial degradation through hydrolysis of *P*-*O*-alkyl and *P*-*O*-aryl bonds is considered the most significant step in detoxification. Analogous phospho-monoesterase and diesterase, which degrade methyl and dimethyl phosphate, respectively, have been reported in bacterial strains like *Klebsiella aerogenes* (Wolfenden and Spence, 1967) and are produced only in the absence of inorganic phosphate from the growth medium. The final enzyme in the postulated degradative pathway is bacterial alkaline phosphatase, which can hydrolyze simple monoalkyl phosphates and is also regulated by the level of phosphate available to the cell (Kertesz *et al.*, 1994).

The hydrolysis of linuron by the enzymes could follow two possible mechanisms. Attack at the linkage between the carboxy group and the 1-methyl, 1-methoxy-substituted nitrogen would yield the corresponding phenylcarbamic acid. This compound is unstable and disintegrates spontaneously to the halogen-substituted aniline and CO_2 (Wallnofer *et al.*, 1970). The amide linkage between the aniline and the carboxy group would give rise to the immediate formation of the halogen-substituted aniline and N-methyl-N methoxy-carbamic acid, N, O dimethylhydroxylamine and CO_2 are also formed as degradation products.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site and survey for pesticides applications in horticultural farms

Four regions of Central and Rift-valley Kenya namely Thika, Kirinyanga, Naivasha and Gilgil formed areas of study. Thika is located at 1^{o} 3′ 0″ south, 37^{o} 5′ 0″ East with a variety of soil type that included aerosols, alisols, lixisols and luvisols. Kirinyaga region is located at 0^{o} 10 ′ 0″ south, 37^{o} 18 ′ 0″ East and two soil types planosols and vertisols were dominant. Gilgil is situated between Nakuru and Naivasha with the following geographical coordinates 0^{o} 13′ 0″ South 36^{o} 16′ 0″ East. Soil types in the region were mainly andosols and fluvisols. Naivasha is northwest of Nairobi in the scenic Rift Valley and flower farming's epicenter. It is located 0^{o} 43′ 0″ South 36^{o} 26′ 0″ East with cambisols and arenosols types of soils.

Information regarding the crop grown, agrochemical used, target pest for each, pesticide trade name and class of pesticides being used in the farms, the period of usage of a particular pesticide as well as their effect on the crop and the type of crop grown was obtained using a questionnaire (appendix 1).

3.2 Collection of soil samples

Sampling was done using stratified random sampling method from in eight farms from the two regions. The geographical regions formed four strata namely; Thika, Kirinyanga, Naivasha and Gilgil. From each region, two plots were identified based on the type of horticultural crops grown in the farms. 200g of the upper top soil (5 cm deep) mineral soil was collected cross-sectional within a given plot hence obtaining nine soil samples from each plot. The soil samples were mixed to form a composite sample and placed in sterile polythene paper. Similar sampling was done 100m away were no horticultural activities took place to act as controls; the samples were placed in cool box, transported to the laboratory and stored at 4^0c.

3.3 Growth medium for isolation of diazinon and linuron degrading bacteria

Mineral salts medium (MSM) was prepared by weighing the salts as described by (Boon *et al., 2000).* the medium contained 1,419.6 mg of Na_2HPO_4, 1,360.9 mg of KH_2PO_4, 98.5 mg of $MgSO_4$, 5.88 mg of $CaCl_2 \cdot 2H_2O$, 1.16 mg of H_3BO_4, 2.78 mg of $FeSO_4 \cdot 7H_2O$, 1.15 mg of $ZnSO_4 \cdot 7H_2O$, 1.69 mg of $MnSO_4 \cdot H_2O$, 0.38 mg of $CuSO_4 \cdot 5H_2O$, 0.24 mg of $CoCl_2 \cdot 6H_2O$, 0.10 mg of MoO_3, and 3.2 mg of EDTA in 1 liter of distilled water Soil samples (1 g) was dissolved in 9 ml of sterile 0.85% NaCl and serially diluted to dilution 10^{-6}.

3.4 Enrichment of soil samples with the pesticides and bacteria isolation

Linuron which was 99.9% pure from Fluka and Diazinon 99.8% pure from Supelco both at a concentration of 10,000 mg /l was used. A stock solution of 500 mg /l equivalent to 500 PPM was made in methanol by dissolving 50ml into 950 ml of distilled water for both pesticides. To make a working concentration of 50 ppm of linuron 10ml was picked from the stock solution and added to 90 ml of MSM in 200 ml Erlen–meyer flasks. To make 12ppm working concentration of diazinon 2.5 ml were and dissolved in 97.5 ml of water and aliquots of 1.0 ml from dilution 10^{-3} from each soil sample was inoculated in both flasks of linuron and diazinon and incubated in a shaker incubator shaking at 12000rpm. All the experiments were done in duplicates. The controls were uninoculated medium with linuron and diazinon; and inoculated medium without the pesticides. Growth in the flasks was monitored periodically by measuring changes in cell turbidity (OD_{600}). After the optical density reading indicated that growth of the cultures was constant, aliquots of 0.1 ml from enriched cultures of the soil sample were streaked on MSM medium supplemented with either Diazinon, or Linuron and 2% agar. The plates were incubated at 28^{o}C for 14 days and distinct single colonies were picked.

The colonies were precultured in Erlenmeyer flasks containing fresh mineral salts medium supplemented with 50 ppm and 12 ppm linuron and diazinon pesticides respectively. Flasks were incubated at 28^{o}C with shaking (1200 rpm) in the dark. Growth was monitored as changes in turbidity (OD_{600}) on a Spectrophotometer (Pharmacia Biotech Novaspec II, 1996 model). HPLC (Shimadzu, 2002 model)

was used for monitoring pesticides degradation There were two controls, one consisting of uninoculated medium with the pesticides Linuron and Diazinon and inoculated medium without Linuron and Diazinon (Sorensen and Aamand, 2003).

3.5 Preparations of standard curves for diazinon, linuron and phosphatase assay

Standards of both pesticides were used to plot standard curves by making dilution ranging from 10 to 50ppm for linuron and 0.5- 12 ppm for diazinon (Appendix 3a and b). 20 µl of each dilution was injected into the HPLC machine for quantification. In Protein analysis, modified lowry protein assay standard curve was prepared using Bovine serum albumine at triplicate points of 0, 20, 40, 60, 80, 100 and 120µg and turbidity measured at OD 660 nm (appendix 3b). The data was fit with a linear regression by the line y = 262.25x – 1.3095 with an R2 value of 0.987 (appendix 3c). Phenolate assay standard curve was produced using p-nitrophenol at triplicate points of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg optical densities determined at OD 405nm.

3.6 Cultural and biochemical characterization of bacteria

3.6.1 Cultural and microscopic examination

Preliminary characterization was performed using morphological and cultural characteristics as described by (Holt *et al.,* 1994). Morphological identification of the isolate was done under the dissecting and compound microscope supplemented

with classical gram staining method. Bacteria strains were cultured on MSM supplemented with diazinon and linuron as carbon sources, after cultivation bacteria colonies were transferred to a glass slide and heat fixed. Crystal violet stain was applied to the heat fixed smear on the glass slide and rinsed with water.

Blotting was done to the smear and examined under a microscope x40. The stain colored the bacteria cells so that their size, shape and arrangement could be observed.

Gram staining was done to differentiate between a gram positive and gram negative bacteria. After heat fixing the bacteria smear on the glass slide crystal violet was added and rinsed off with water. Gram iodine was added and rinsed off with water, and then the smear was decolorized with 95% ethanol and counterstained with safranin. Bacteria that retained the primary stain during alcohol decolorization are considered gram positives and that that losses the primary stain are referred to has gram negatives (Alexander and Strete 2001).

3.6.2 Biochemical characterization of diazinon and linuron degrading bacteria

To assess the ability of isolates to break down different compounds with similar chemical bonds to the pesticides under study, biochemical test were carried out using several culture mediums composed of different organic compounds. Results from the tests were also significant in presumptive identifications of the isolates prior to phylogenetic analysis.

3.6.2.1 Glucose oxidation fermentation (O-F) test

Glucose oxidation test was done using Hugh and Leifon's oxidation fermentation (O-F) media (pH 7.1) containing bromothymol blue indicator based on Harold (2002) protocol. After inoculation of bacteria isolates one set of the tubes was covered with hot liquid Vaseline, which solidified to provide an anaerobic environment and the second set left open. Following 24 hr incubation at 28^0C acid production from glucose was indicated by color change from blue to yellow. In the oil covered tubes strict aerobes do not show any color change in the medium while facultative anaerobes ferment glucose characterized by acid production and color change. in open tubes oxidation occurs and acid production causes color change (Cappuccino and Sherman, 2002).

3.6.2.2 Methyl Red-Voges- Proskauer (MR-VP) test

MR-VP test was used to determine the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products according Harold's (2002) protocol. MR-VP broth was inoculated with each of the isolates and incubated at 27 °C for 72 hours. 2.5 ml transferred to a new tube 5 ml of pH added to the tube Methyl red indicator and if the medium turn red the isolate uses mixed acid pathway to metabolize pyruvic acid to other acids such as lactic and formic acid. Bacteria that metabolize pyruvic acid to neutral end product like acetoin lower the pH to 6.0 and at this pH methyl red is yellow in color showing negative results. (Cappuccino and Sherman, 2002).

3.6.2.3 Gelatin liquefaction

Nutrient gelatin was used to demonstrate the hydrolytic activity of gelatinase (Harold, 2002). After inoculation of the bacteria using sterile wire loop incubation is done at 28 ^{o}C for 24 hrs. Cultures are placed in the refrigerator at 4°C for 30 minutes and cultures that remained liquefied after chilling were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

3.6.2.4 Indole production

Sulfur-Indole Motility (SIM) agar media was used to test the production of tryptophanase enzyme and the ability to produce hydrogen sulfide from substrate such as sulfur containing amino acids and organic sulfur after inoculation of bacteria using a stile wire loop. Presence of indole was detected by addition of Kovac's reagent to 48-hour cultures of each isolates (Harold, 2002). Positive results were indicated by production of a cherry red layer. Absence of black coloration in the media following incubation indicated absence of hydrogen sulfide (Cappuccino and Sherman, 2002).

3.6.2.5 Catalase test

Catalase production was determined by addition of 3% hydrogen peroxide to Tryptic Soy Agar (TSA) cultures of each isolate based on the methods outlined by Cappuccino and Sherman (2002). A positive reaction was indicated by formation of bubbles, which indicated break down of hydrogen peroxide to water and oxygen.

3.6.2.6 Oxidase test

Cytochrome oxidase production was determined using oxidase strips containing tetramethy-p-phenylenediamine dichloride. Blue purple coloration indicated a positive test after a 24 hrs bacteria colony was smeared over the strip (Harold, 2002).

3.6.2.7 Nitrate reduction test

The ability of the isolates to reduce nitrates to nitrites was carried out using nitrate broth medium containing 1% potassium nitrate according to the methods of Harold (2002) Following inoculation and incubation, addition of sulfanilic acid and alpha-naphthylamine produced a cherry red coloration, which was indicative of positive results (Cappuccino and Sherman, 2002).

3.6.2.8 Urease test

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using urea broth media containing phenol red indicator. A positive reaction was indicated by development of deep pink color when pH is lowered by ammonium compounds to 8.4 (Cappuccino and Sherman, 2002).

3.6.2.9 Phosphatase enzyme activity assay

Alkaline phosphatase activity was measured spectrophotometrically by monitoring the release of para-nitrophenol from paranitrophenyl phosphate (PNPP) at 400 nm.

A typical assay contained 200 mM sodium glycine buffer at either pH 9.0 or 10. 5 mM calcium chloride, 330 Mmol of PNPP, and an appropriate amount of spent culture medium, in a final volume of 1 ml. Reactions were performed at room temperatures and stopped by addition of 50 ml of 4 M sodium hydroxide. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 mM PNPP per min (Bolton and Dean 1972). Protein concentration was determined by modified method of (Lowry *et al.*, 1951) Activities were expressed as unit per milligram of substrate oxidized per ml of protein.

3.6.2.10 Esterase enzyme activity assay

The enzymatic activity was performed by initially growing the isolates in BHI (brain heart infusion) broth for 24 h. at 30ºC. After that, aliquots of 100 ml were inoculated on the media as described by Sierra (1957), containing (g l-1): peptone 10.0, NaCl 5.0, $CaCl_{22}H_2O$ 0.1, agar 18.0, pH 7.4. To the sterilized culture media, previously sterilized Tween 80 was added in a final concentration of 1% (v/v). The cultures were incubated at 30ºC during 96 h. The Enzymatic Index (EI) was determined within 24, 48, 72 and 96 h. The Enzymatic Index (EI) was expressed by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth (Hankin and Anagnostakis, 1975).

3.7 Chromatographic characterization of bacterial isolates

3.7.1 High pressure liquid chromatography pesticides degradation analysis

The degradation of the pesticides was monitored by high performance liquid chromatography. (Shimadzu HPLC class *VP* series) with two LC – 10 AT *VP* pumps variable wavelength UV detector SPD10*VP* (Shimadzu) and a reverse phase C-18 column,250 x 4.6mm,fitted with a C-18 silica reverse phase guard column was used. (Fisher Scientific, Fairlawn, N.J) The HPLC system was equipped with software class *VP* series ss420x (Shimadzu).

The mobile phase components acetonitrile and degassed water were pumped from the solvent reservoir to the column at a flow level 1 mL/min. The column temperature was maintained at 27^0 C. 50 µL of the cultural samples and control were removed periodically and supernatants of bacterial cultures were analyzed by a reverse phase HPLC. Cells were removed by centrifugation (10 min at 5000 X g) and 20 µL of sample was injected using Rheodyne syringe. Compounds were identified by their retention times and peaks area corresponding to reference standards and reduction in concentration calculated using the equation Y= mx+c

3.7.2 Gas chromatography –mass spectrometry analysis of degradation metabolites.

Cultures that showed decrease in concentration in HPLC were further analyzed by GC-MS to identify the metabolites of degradation. Liquid samples (10 ml) were

fulfilled to volume of 50 ml with deionised water and extracted (2x with) with 20ml hexane on a rotary shaker for 1 h. next, the extracts were dehydrated with anhydrous Na_2SO_4 and evaporated to dryness under a stream of N_2 at 45^0 C using a rotary evaporator (IKA, RV Basic, Janke and Kunkel-labortechnik, Germany) and subsequently diluted to a final volume of 10 ml with acetone and reserved for chromatographic analysis.

Analyses were performed using a GC-MS 7890A stand-alone gas chromatograph (Agilent Technologies, Inc., Beijing, China) and a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following conditions: Inlet temp 270°C, transfer line temp of 280°C, and column oven temperature programmed from 35 to 280°C with the initial temperature maintained for 5 min then 10 °C/min to 280 °C for 10.5 min and the final one for 29.9min 50 °C/min to 285 °C(run time 70 min). The GC was fitted with a HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25-μm) (Restek, Bellefonte, PA, USA). Helium at a flow level of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temperature of 250°C and a quadruple temperature of 180°C. 230°C was set as the MS ion source temperature. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV.

Aliquots of 1.0 μL extract were automatically injected in the split/ splitless mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40-550 m/z mass range in the full scan. The filament delay time was set as 3.3 min. Library –ms searches NIST/EPA/NIH MASS SPECTRAL

LIBRARY (NIST 05) and NIST MASS SPECTRAL SEARCH PROGRAM Version 2.0d, chemco and adams data base were used for characterization purposes in the GC-MS data system.

3.8 Molecular characterization of bacterial isolates 16S rRNA

3.8.1 DNA extraction

Pure cultures of the isolates were inoculated in 20 ml of freshly prepared nutrient broth in universal bottles and incubated for 24 hrs in a shaker incubator at 30^{o}C and 200 rpm. One and half of the culture was added to eppendorf tube and centrifuged at 13000rpm for 5 minutes, the supernatant was discarded. One milliliter of TE buffer was added and the above step repeated to gather enough cells. The pellet was re-suspended in (200 µl) of solution 1 (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25% sucrose solution). Five (5µl) of lysozyme (20 mg/ml) was added and contents gently mixed. Five microlitres of RNase A (20 mg/ml) was added and gently mixed. Incubation was done at 37^{o}C for 1 hour. Following incubation, (600µl) of solution 2 (10 mM Tris pH 8.5, 5mM EDTA pH 8.0 and 1% SDS) was added and contents were mixed by inverting the eppendorf several times. (10µl) of proteinase K (20mg/ml) was added and mixed gently followed by incubation at 50^{o}C for 30 minutes. DNA was extracted by adding equal volumes of phenol: chloroform and centrifuged for 20 minutes at 13000 rpm and carefully pipetted out the aqueous phase which contained the crude DNA. The aqueous phase was transferred to a new tube and DNA was stabilized by addition of 0.1 volumes

of 3M NaCl and precipitated with an equal volume of absolute ethanol (Sambrook *et al*., 1989).

DNA was then stored at -80^{0}c over night. Genomic DNA was centrifuged and the pellet was rinsed with 70% ethanol to remove traces of salt. The DNA pellet was air-dried for 1 hour to completely remove the ethanol then re-dissolved in (200µl) of TE and stored at –20^{o}C. The DNA was semi quantified on a 1% agarose 1X TAE buffer and visualized under ultraviolet by staining with ethidium bromide (Sambrook *et al*., 1989).

3.8.2 PCR amplification and purification of bacterial 16S rRNA

Bacterial genomic DNA was extracted from (100 mL) of bacterial cultures as described by (Sambrook *et al*., (1989). The 16S rRNA gene of the strain was amplified by PCR using the primer set 27f (5'-AGTTTGATCCTGGCTCAG-3') and 1492(5'GTTACCTTGTTACGACTTC-3') (Sigma Aldrich) under standard PCR conditions. The amplified fragment was purified by a QlAquick spin PCR purification kit (QIAGEN GmbH, Hilden, Germany). PCR products were visualized using a gel electrophoresis in 1.0% agarose gel.

The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen, Germany) according to manufacturer's instructions. Five volumes of buffer PB (Qiagen, Germany) was added to 1 volume of the PCR sample and thoroughly mixed. The QIAquick spin column was placed in a 2 ml collection tube, the sample applied to the QIAquick column to bind the DNA, and then centrifuged

for 30-60 seconds at 13000 rpm. The flow-through was discarded, and the QIAquick column placed back into the same tubes. To wash the DNA, 0.75 ml buffer PE was added to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the column centrifuged again for an additional 1 minute at 13000 rpm to remove residual ethanol from buffer PE. The Qiaquick column was placed in a 1.5 ml microcentrifuge and thirty (30µl) of buffer EB (10mM Tris-Cl, pH 8.5) added to elute DNA. The tubes were then centrifuged for 1-minute, the spin column removed and DNA stored at -20^{o}C for application (Sambrook *et al.*, 1989).

3.8.3 Restriction fragment length polymorphism (RFLP) and sequencing of PCR products

The PCR products were digested by restriction endonucleases Hha1 from *E.coli* that carries a gene Hha1 from *Haemophilus haemolyticus* (ATTC 10014) 20,000 units/ml concentration. Enzyme 0.2 µl, buffer 3 µl, PCR product 7 µl, and the reaction volume topped up to 30 µl, with double distilled water and incubated in water bath at 30^{0}.c for 1 hrs.Purified PCR products were cycle- sequenced with an ABI prism big dye ^{TM} terminator, cycle sequencing ready reaction kit (Perkin-Elmer) 60 ng template DNA, 2 pmol primer 27f, 2 ul of nucleotides, enzymes and cofactors in a reaction tube (10 ul). Unincorporated dye terminator was removed using QIGEN DYE EX ^{TM} spin kit and products analyzed using an ABI prism 310 DNA sequencer (perkin Elmer). This was done at ILRI (a commercial service provider)

3.8.4 Phylogenetic Analysis of the DNA sequences

The sequences were compared to sequences in public databases with the BLAST search program on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nml.nih.gov/blast) to find closely related bacterial 16S rRNA sequences. The MEGA 4 software package (Tamula *et al,* 2007) was used to align the sequence. Highly variable regions of the 16S rRNA gene sequence and sequence positions with possible alignment errors were excluded by only using those positions of the alignment that were identical in at least 50% of all sequences. Sequence similarity matrices and phylogenetic tree was calculated and visualized using Tree View of the same software according to neighbor joining method and maximum-likelihood (Thompson *et al.*, 1994).

3.9 Data analysis

Data from repeated experiments was subjected to analysis of variance (ANOVA) for each treatment and means separated using LSD using the SAS (SAS/IML software; Version 9.1; SAS Institute 1999) program. Regression analysis was used to determine the relationship between total protein and phenolate liberation, Probability value of $P<0.05$ was used for entire tests to show statistical significance of mean values for parameter analyzed.

CHAPTER FOUR

4.0 Results

4.1 Pesticide use in horticultural farms

Frequency of pesticides applications on farms under horticultural production surveyed revealed the use (36) different formulations. The study showed that of the different pesticide formulation types used by farmers in the four regions sampled (22) of them were pesticides and (14) were herbicides. In the pesticide category diazinon had the highest proportions of application at 45.9 % in the four regions. Gilgil region recorded the highest level of diazinon usage at 13.9% proportion of application (Figure 5). Demikill was ranked the second with an application proportion of 18.9% in the four regions. Gilgil recorded the lowest proportion of diazinon usage at 2.8%. Brigade, Furadan, Methyl bromide and Folicur recorded the lowest level of application at 5.5 % in the study. Within the four regions surveyed, the proportion of pesticide usage was highest in Gilgil region at 29.1 % proportion of application, followed by Naivasha at 28.3 %, Kirinyaga 23.5% and the least in pesticide application level was Thika region at 19.1%.

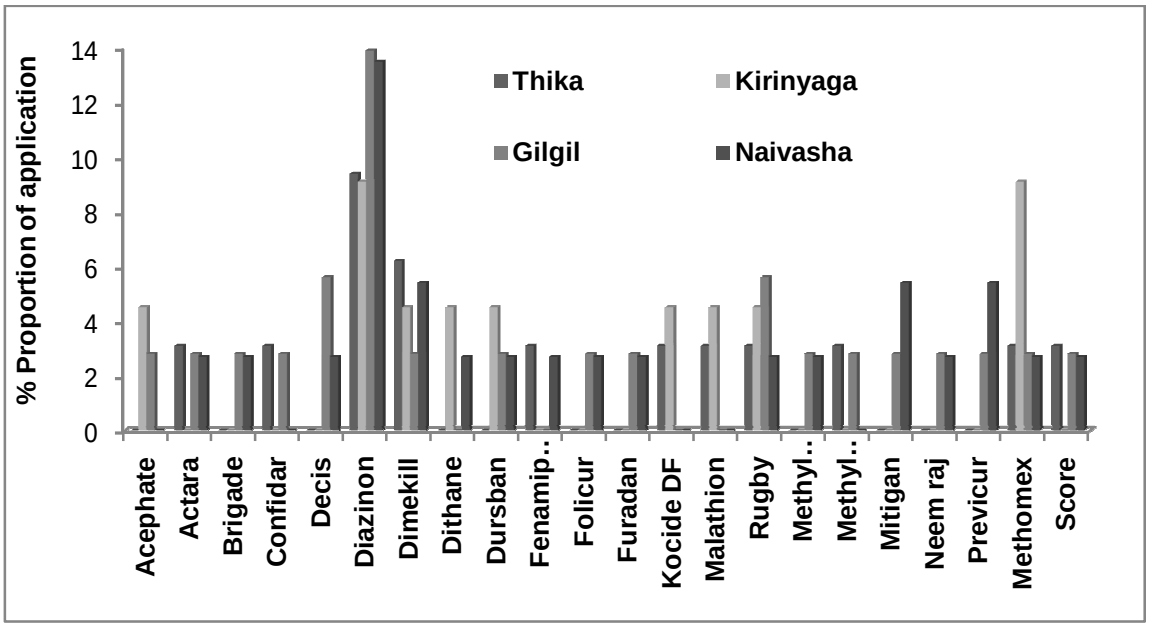

Figure 5: Proportion of pesticides applied by farmers

Among the (14) different herbicide formulations Linuron recorded the highest level of application of 31.6 % in the four regions sampled (Figure 6). Isoproturon followed in application with a proportion of 21.6 % of application. Neprapamide had the lowest proportion of application with 1.6 % ratio of use compared to other hebocides. Pesticides classification into their respective chemical groups placed them into 7 groups (Figure 7). The group organophosphates were ranked the highest in usage in four regions. Gilgil had the highest application proportion of 33.08 % followed by Thika region with 27.06 %, and Naivasha 22.55 %. In Kirinyaga organochlorides group was highly used with a 63.26 % proportion of application. In the herbicides category the phenylurea group was highly applied in Gilgil at 36.73 %, followed by Thika 28.57 %, Kirinyaga 18.36 % and Naivasha at 16.32 %.

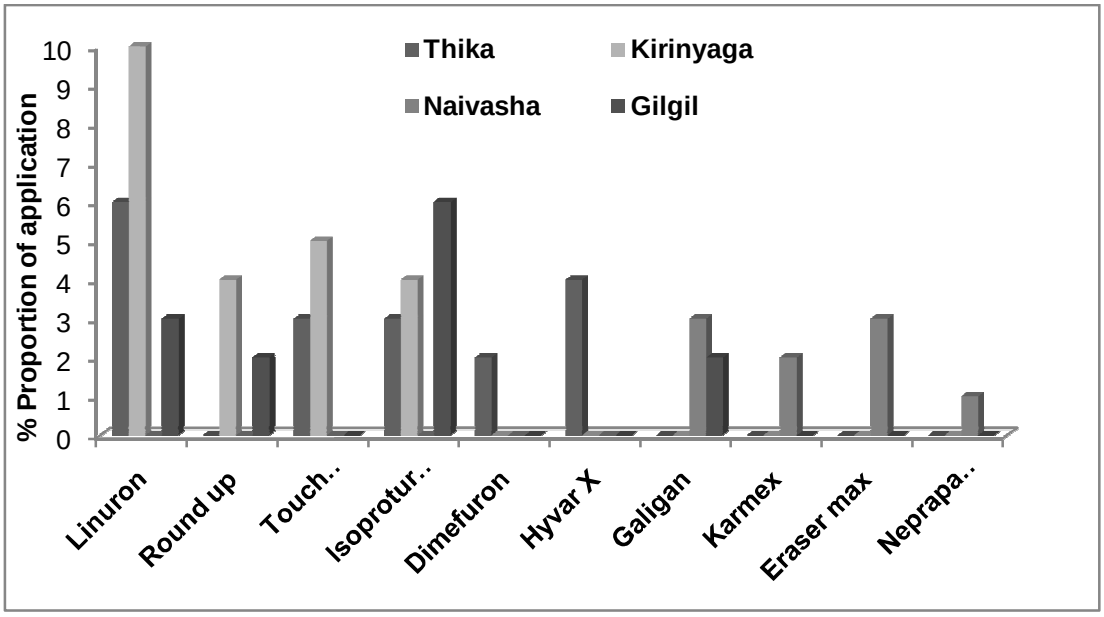

Figure 6: Proportion of herbicides applied by farmers

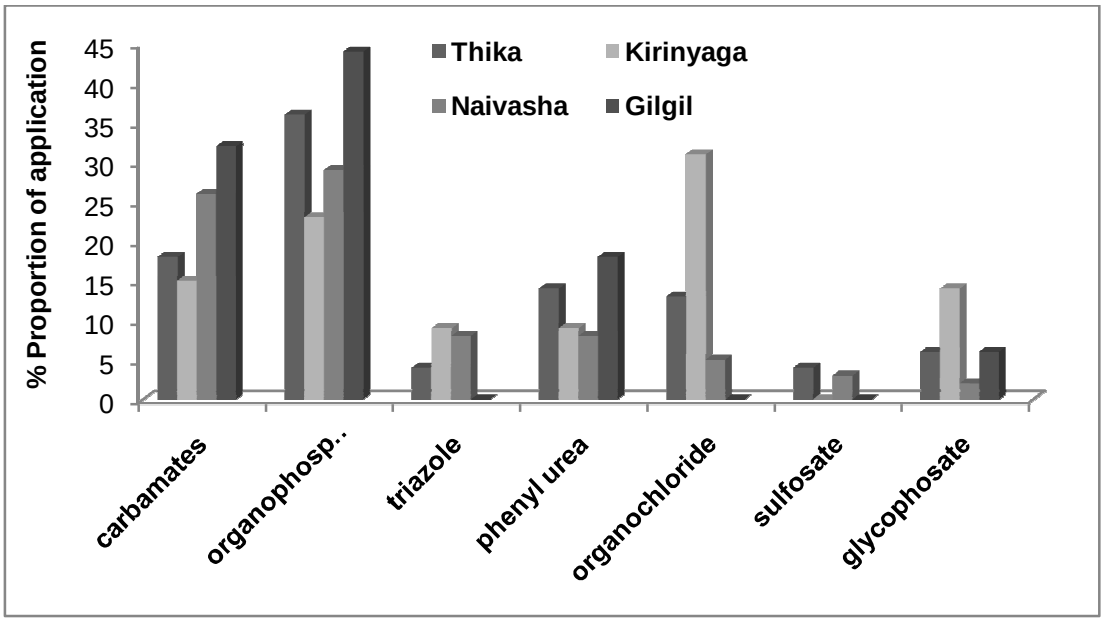

Figure 7: Pesticides classification on the basis of functional groups on their molecular structure

4.2 Diazinon and linuron soils enrichment cultures

In the diazinon enriched soil cultures the concentration of 12ppm diazinon decreased drastically in the initial 9 days of incubation (Figure 8a) however, increase in cell mass OD_{600} in both flasks of diazinon soil enriched and non diazinon soil enriched cultures was minimal ranging between 0.05-0.0015. On the 6^{th} day cell mass in the diazinon enriched soil cultures increased to OD_{600} of 0.2 while the non enriched cultures recorded a low cell mass of 0.002. On the 9^{th} to the 12^{th} day cell mass in the diazinon enriched soil culture flask increased to OD_{600} of between 0.5 -0.7 while the non enriched culture decreased in cell mass. Linuron enrichment cultures had a longer lag phase of 7 days (Figure 8b). Cell mass OD_{600} was at a range of 0.005-0.063 for both enriched and non enriched cultures. On the 14^{th} day cell mass in the linuron enriched soil culture flasks cell mass increased significantly to OD_{600} of 0.78 and a reduction of linuron concentration by 13% to 43 ppm. By the 21^{st} day of incubation cell mass had reached OD_{600} of 1.08 and stabilized at this point reducing linuron concentration by 36% to 32 ppm. Over the 35 days of incubation the cell mass of the linuron non enriched soil culture flasks remained low with a maximum cell mass OD_{600} of 0.97 these observations clearly demonstrated that there were positive interactions between pesticide enriched cultures and cell mass increase.

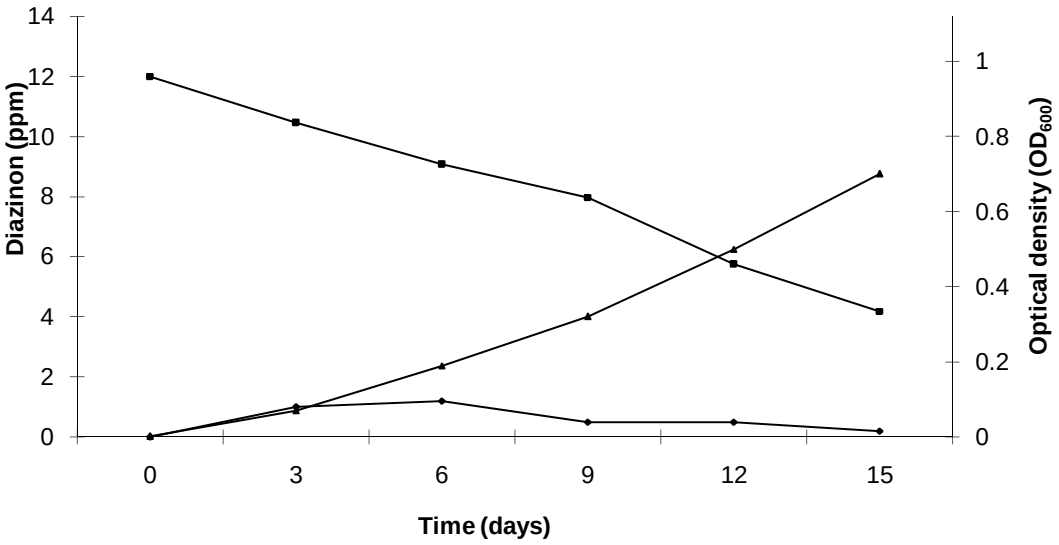

Figure 8a: Microbial growth curves of diazinon enriched soils. The symbols represent ■ Diazinon concentration in enriched soils, ▲ growth curve for Diazinon enriched culture, ♦ growth curve for non enriched soil cultures.

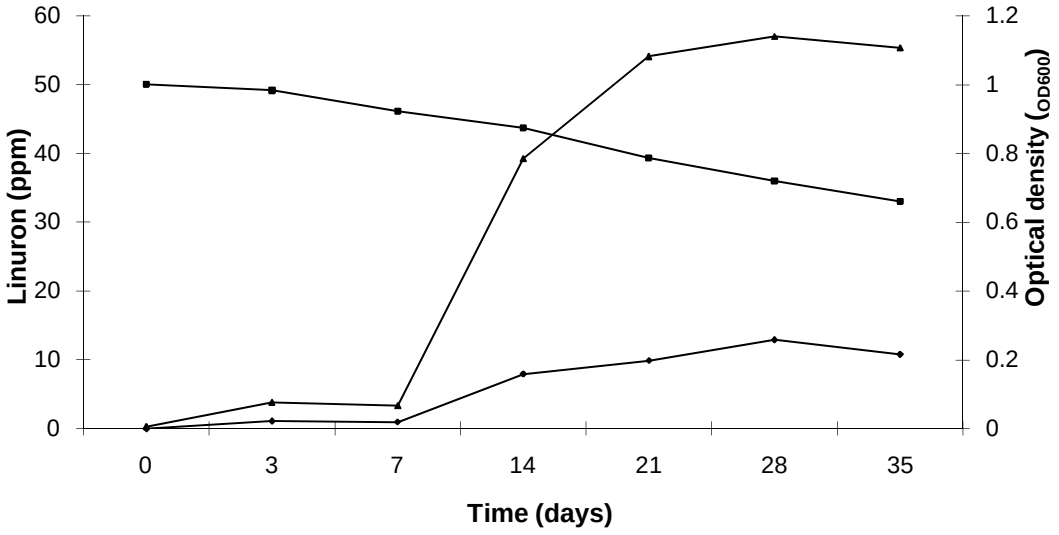

Figure 8b: Microbial growth curves of linuron enriched soils. The symbols represent ■ linuron concentration in enriched soils, ▲ growth curve for Linuron enriched soil culture, ♦ growth curve for linuron non enriched soil cultures.

4.3 Cultural and isolation of diazinon and linuron degrading bacteria

Using two enrichment cultures steps a total of thirty one (31) different strains were isolated from soils sampled in the four different regions. Eighteen (18) of the isolates were diazinon degraders and assigned codes (Table 1) while thirteen (13) isolates were linuron degrading bacteria. Out of the thirteen (13) linuron degrading bacteria, 6 isolates were from Thika soils (47% of the active isolates), from Naivasha (29%), and Gilgil (18%), and Kirinyaga region (6%). From soils of Thika region 8 diazinon degrading isolates were isolated (38% of the active isolates), Naivasha (24%), Gilgil (19%) and Kirinyaga region had (19%).

Table 1: Total number and codes of diazinon and linuron degrading bacteria isolated from each site

Site	Diazinon total Isolates	Diazinon Isolate codes	Linuron total Isolate	Linuron Isolate codes
Thika	8	Dwa-7B, DGa-2A, DWa-7A, DGa-2C, DGa-2B, DJk-4A, DJk-4B, DJk-4C	6	Lwa-2C,LGa-4B,LJk-5A,LJk-5B,LJk-5C, LWa-2A,
Kirinyaga	2	DKi-6A and DKi-6B,	1	LKi-6A,
				LSh-6B, LOG-8A
			4	LSh-6C,LSh-6A,
Naivasha	3	DSh-3C, DSh-3A, DLOG-8A		
Gilgil	5	DLla-1A, DLla-1B, DKa-5A, DKa-5B, DKa-5C,	2	LLla-1A and LKa-7A

4.4 Chromatographic profiles of diazinon degradation by bacteria

Diazinon was detected at the 11th minute peak A with the largest area under the curve. Peaks B, C, D and E were also detected as a result of diazinon hydrolysis to give rise to other intermediate metabolites (Figure 9 a). After 15 days of incubation (Figure 9 b) peak A was observed to decrease while the corresponding peaks B, C, D and E were observed to increase in area. On the 25th day of incubation peak A had reduced tremendously and 98.6% of diazinon had been degraded. Peak A was not detected on the 35th day of incubation (Figure 9 d) signifying that the compound was completely degraded by bacterial isolates.

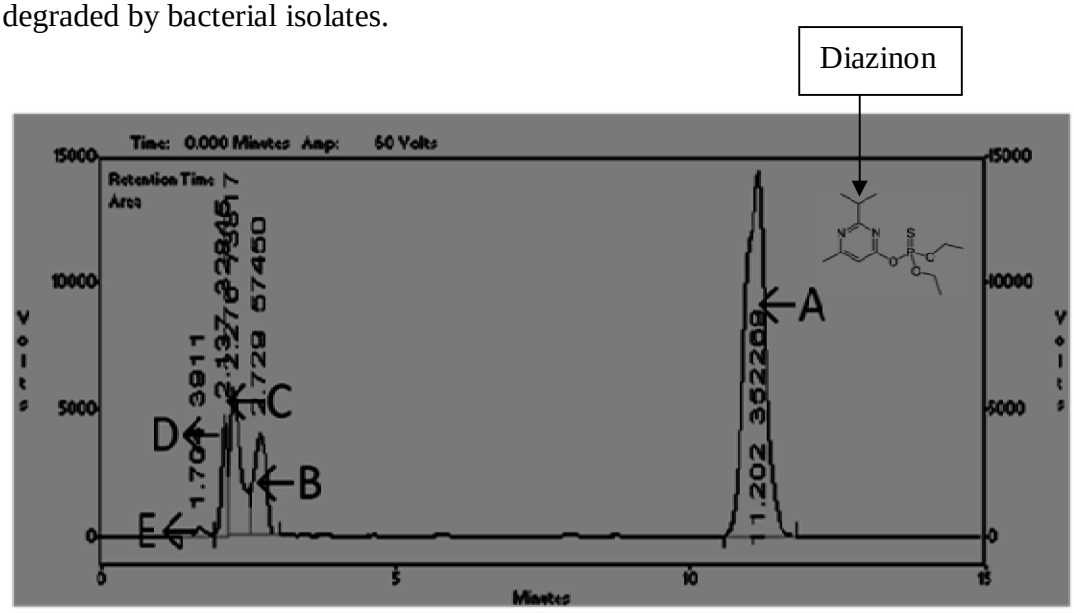

Figure 9a: High pressure liquid chromatography profiles of diazinon and mineral salts at day zero

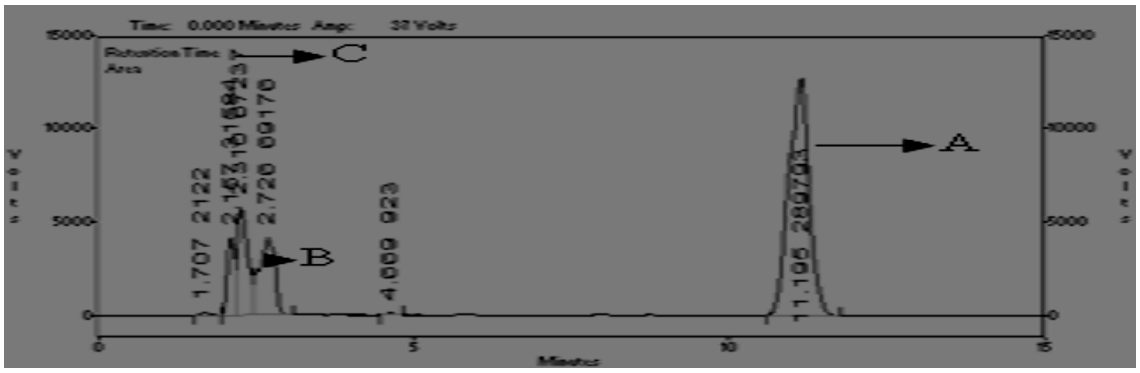

Figure 9b: High pressure liquid chromatography profiles of diazinon degradation at day 15

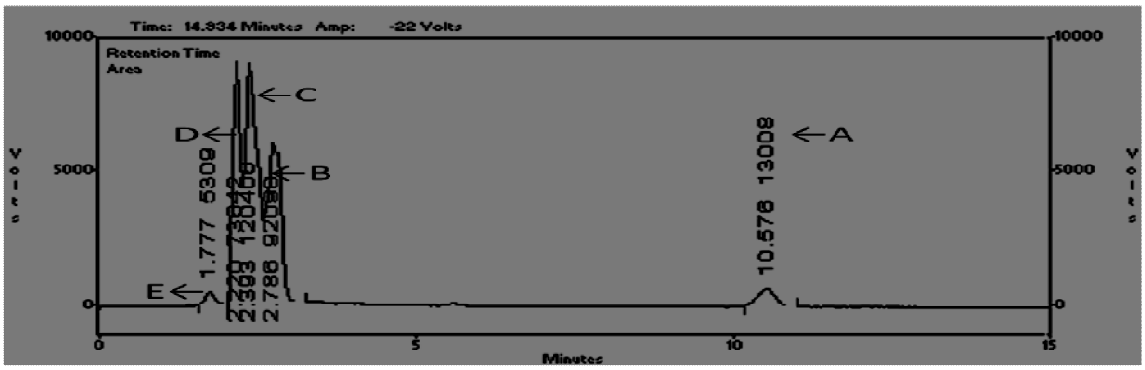

Figure 9c: High pressure liquid chromatography profiles of diazinon degradation at day 25

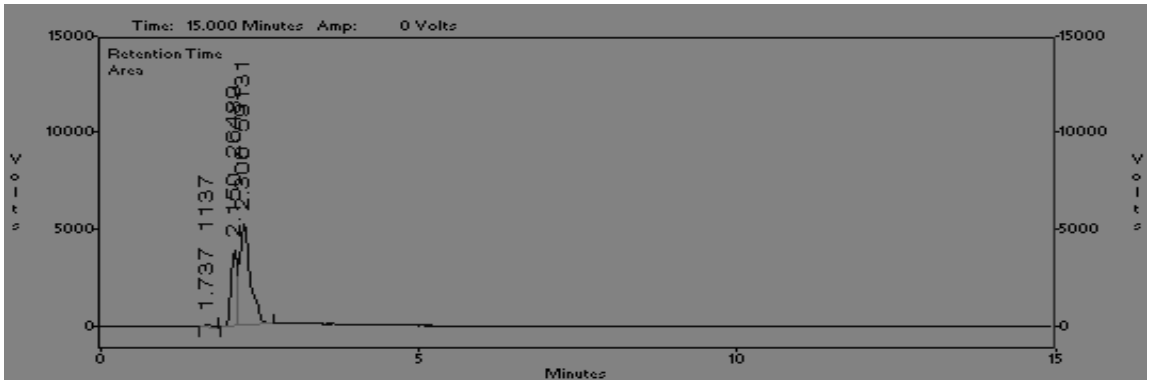

Figure 9d: High pressure liquid chromatograms of diazinon degradation at day 35

4.4.1 Diazinon degradation by soil bacteria

Diazinon degrading isolates were monitored for a period of 35 days (Figure 10a and 10b). Degradation varied significantly with time (days) in all diazinon degrading isolates studied ($P<0.05$). At day 0 the concentration of diazinon in all diazinon enriched flasks together with isolates and the control was not significantly different ($P> 0.05$) however, on the 5th day of incubation the level of diazinon degradation by the six isolates was significantly different ($P=0.01$). Isolate DJk-4C obtained from soils of Thika region had the highest level of diazinon degradation reducing the pesticide by up to 30.7 % followed by isolate DKi-6A isolated (Figure 10b) from the same region with a degradation level of 24.75% and isolate DSh-3C isolated from Naivasha soils was 3rd at a level of 11.15%. Isolate DLoG-8A had significantly lower diazinon degradation level reducing the pesticide by 3.4%. At the 10th day of incubation isolate DJk-4C had the higest degradation of diazinon, reducing the pesticide by up to 43.1%, followed by isolate DJk-4A at 33.6% while isolate DSh-3A had significantly lower diazinon degradation level of 11.5 % compared to other isolates on the 10th day of incubation (Appendix 4).

On the 20th DJk-4A had the highest degradation level of diazinon reducing the pesticide by up to 70% (Figure 10a). The isolate maintained the highest level of diazinon degradation through out the experimental period. 0n the 35th day of incubation isolate DJk-4A had 0.57 ppm of the initial 12ppm of diazinon remaining in the media. Comparing these observations with the negative control which had 9.13 ppm in the

media it was certain that microbial degradation played a big role in diazinon degradation as opposed to abiotic processes. The level of diazinon degradation with time for isolate DJk-4A at the 20^{th} and 25^{th} day was not significantly different ($p= 0.662$). On the 30^{th} and 35^{th} day isolate DSh-3A and DSh-3C had no significant difference in the level of diazinon degradation $P=0.571$ (Appendix 4). Isolate DSh-3C had significantly lower level of diazinon degradation of up to 66.7% at the 35^{th} day.

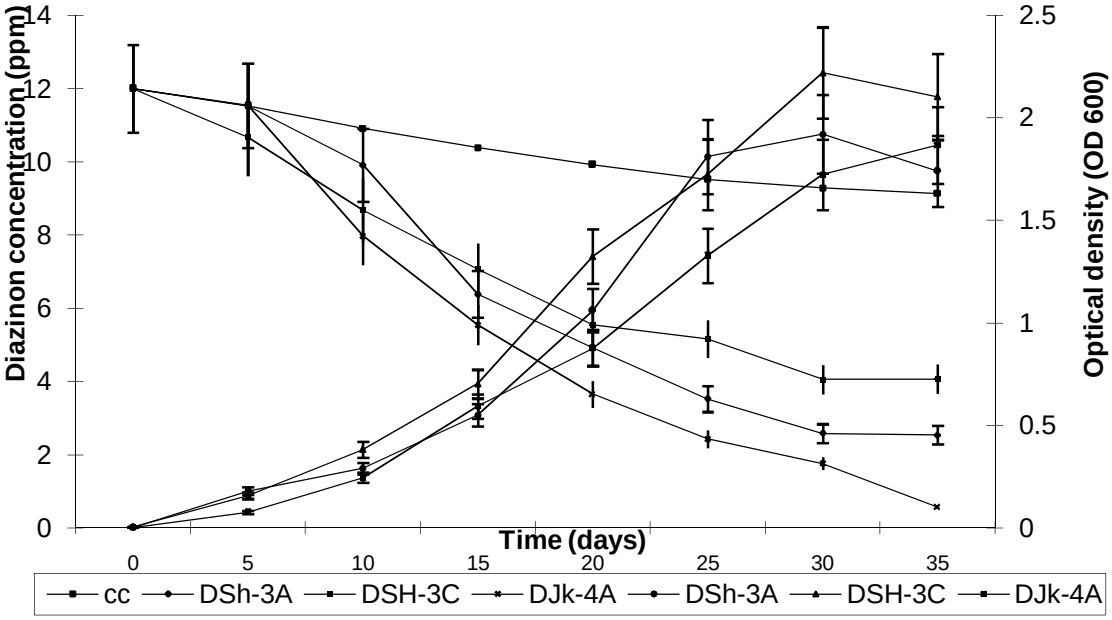

Figure 10a: Degradation of diazinon by isolate DSh-3A, DSh-3C and DJk-4A. The data points and error bars show the means and standard deviations.

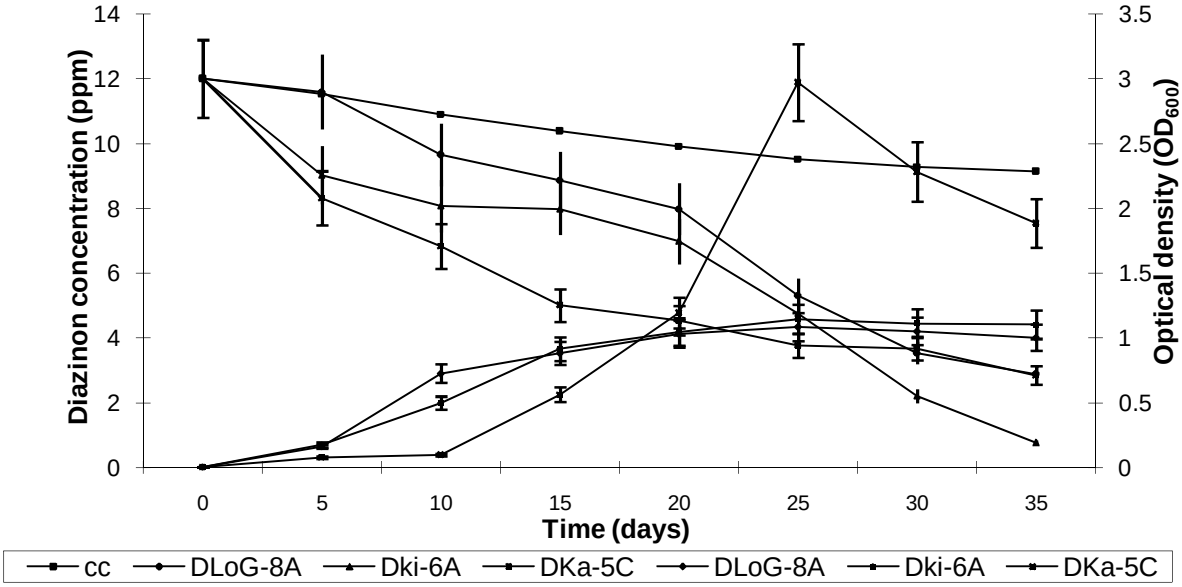

Figure 10b: Degradation of diazinon by isolate DLoG-8A, DKi-6A and DKa-5C. The data points and error bars show the means and standard deviations.

4.4.2 GC-MS identification of diazinon degradation intermediate metabolites

Spectrums obtained from gas chromatography mass spetrometre indicated that diazinon compound was degraded into two intermediate coumpounds (Table 2) in the degradation pathway. The 2-isopropyl-6-methyl-4-hydroxypyimidine (IMHP or oxypyrimidine) is a persistent, less toxic product and demeton-O a product of monoethylthiophosphate oxidation.

Table 2: Main intermediate metabolites of diazinon degradation

Compound Name	Retenti on time	Molecular weight
2 – Isopropyl – 4 – methyl – 6 – hydroxyprimidine	13.586	152
O,O-DiethylO-[2 (ethylthio)ethyl] phosphorothionate (Demeton-O)	26.008	88

4.4.3 Chromatographic profiles of linuron degradation by soil bacteria

Linuron was detected at the 5.4 min (Figure 11a) with the largest area under the curve. Other metabolites were also detected at various retention times 1.8, 2.1, 3.8, and 4.8, with varying areas under their curves. Incubation with isolates LJk-5C, LWA-2C, LLa-1A, LoG-8A, LJk-5B and LWa-2A for 3 weeks at 28^{o} C shaking at 1200 rpm metabolites detected at various retention times B, C and E continued to increase in area while, the area under linuron peak A continued to reduce indicating loss of linuron (Figure 11c) as a result of microbial biodegradation. The trend in reduction of linuron

concentration with time as shown in (Figure 11d) continued while, an accumulation of intermediate metabolites at retention time 1.7, 2.4, 5.0 and 3.8 minutes was observed. Isolates were observed to have preference of metabolites detected at retention time 3.8 and 5.0 minutes to the main compound linuron since these metabolites did not accumulate in the medium throughout the incubation period. In (Figure 11e) several other metabolites were detected at various retention times as the main compound appeared to diminish in concentration.

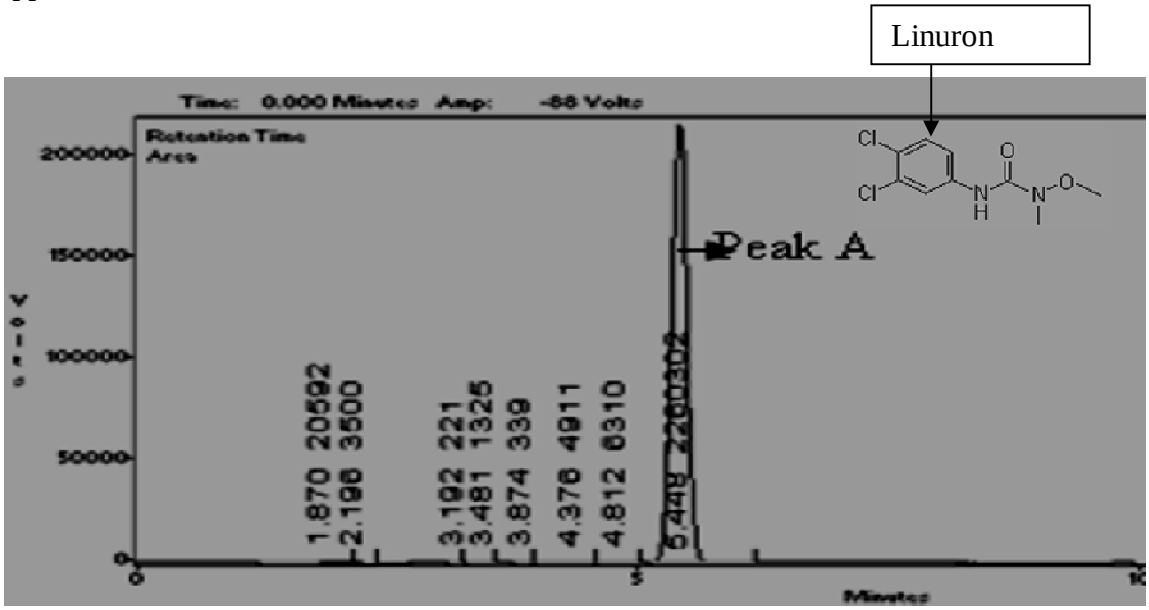

Figure 11a: HPLC profiles of linuron and mineral salts at day zero

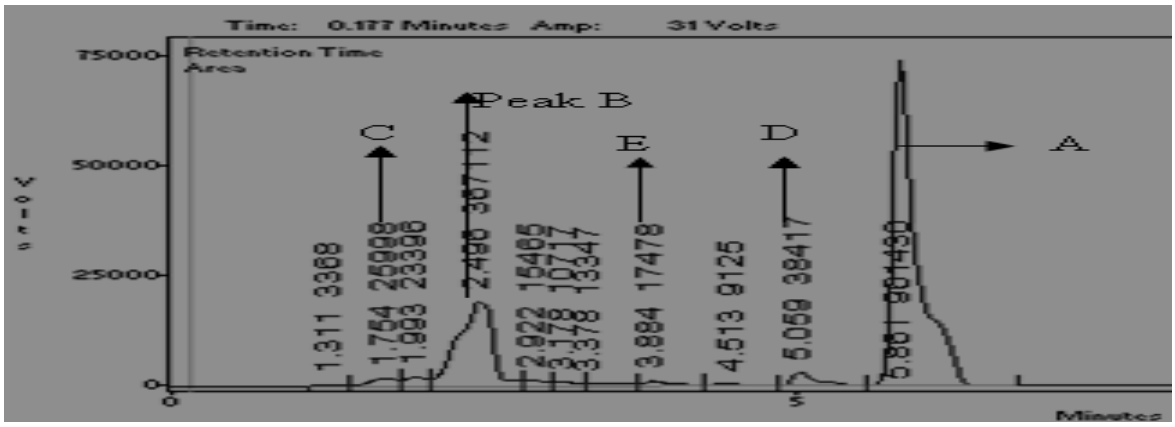

Figure 11b: HPLC profiles of linuron degradation at day 28

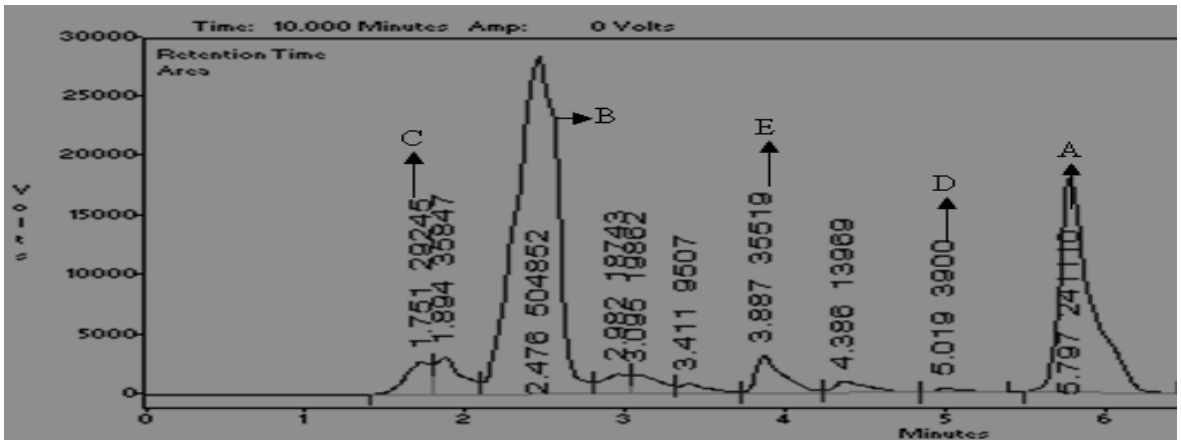

Figure11c: HPLC profiles of linuron degradation at day 42

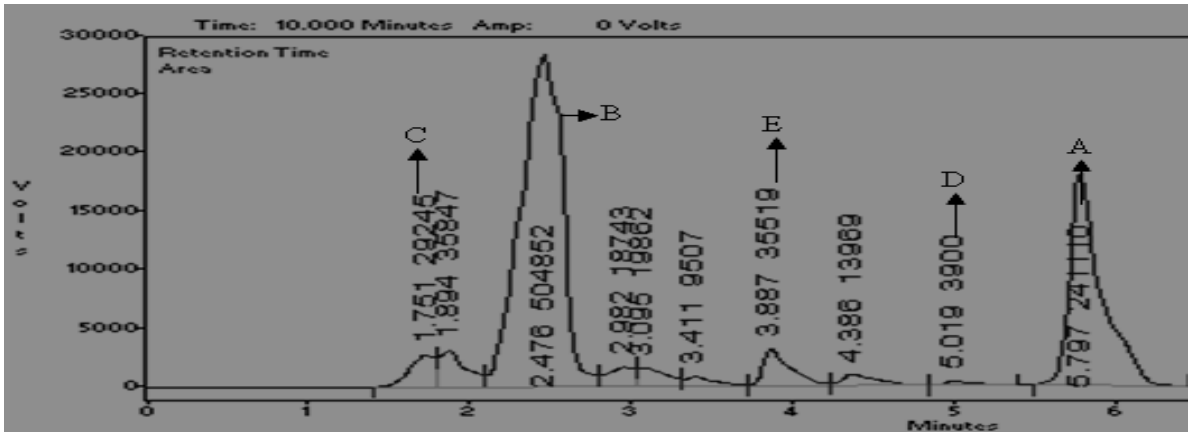

Figure 11d: HPLC profiles of linuron degradation on 63rd day

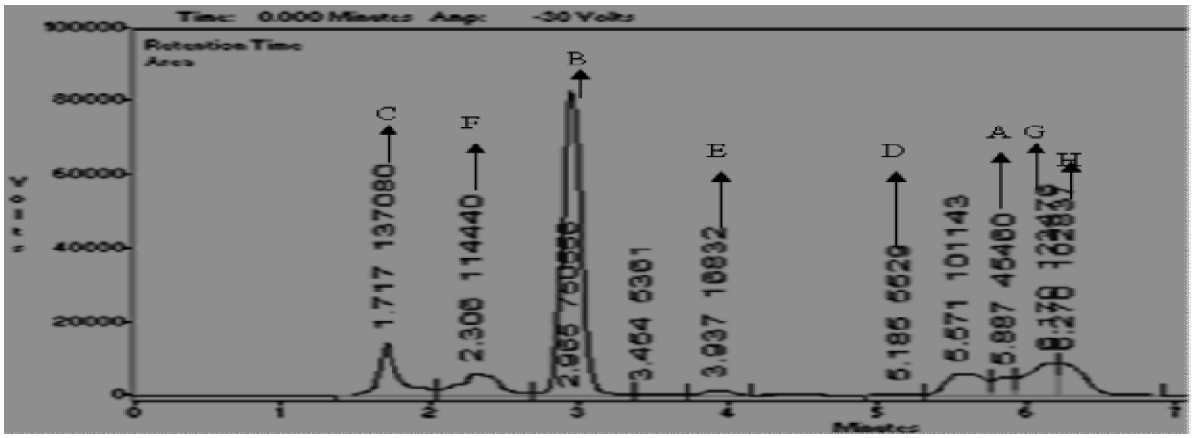

Figure 11e: HPLC profiles of linuron degradation at day 84

4.4.4 Degradation of linuron by soil bacteria

The long lag phase of 7 days (Figure 12a and 12b) was followed by higher growth level of isolates. Linuron degradation varied significantly with time (days) in all linuron degrading isolates studied ($P<0.05$). At 0 days, the concentration of linuron in all 50ppm linuron enriched flasks together with isolates and the control was not significantly different.

On the 7th day of incubation isolates LJk-5B and LJk-5c had the highest degradation of linuron reducing the pesticide by up to 13%. Isolate LlA-1A had the lowest level of linuron degradation of 3% on the 7th day of incubation (Figure 12a) At the 14th day isolates LoG-8A, LWa-2A, LJk-5B and LWa-2C had no significant difference ($P=0.063$) in the level of linuron degradation compared to other isolates (Appendix 5). On the 21st day isolate LJk-5C had the highest degradation level of linuron reducing the herbicide by up to 37.74% while isolates Lla-1a and LoG-8A showed no significant differences

(P>0.05) in the level of linuron degradation had the lowest level of linuron degradation reducing the herbicide by up to 9.58% (Appendix 5).

At the 28th day of incubation isolates LJk-5B and LWa-2A had no significance difference in the level of linuron degradation (P>0.05) reducing the herbicide by up to of 29%. On the 35th day it was observed that the level of linuron degradation by the test isolates was significantly different (P< 0.05) among all the isolates. Isolate LJk-5C had the highest level of degradation reducing the herbicide by up to 55% while isolate LoG-8A was observed to have the lowest degradation ability of linuron of up to 26%. On the 42nd day Isolate LJk-5c was observed to be the fastest linuron degrading isolate with a 69.18% removal level (Figure 12a).

Linuron degradation by abiotic processes was observed in the control flask; on the 56th day concentration of linuron in the control flask was reduced by only 16% while the concentration of linuron at the same test period in the slowest linuron degrading isolate LoG-8A was 46%. Hence it was apparent that the test organisms were contributing to linuron loss from the culture medium (Figure 12b). On the 84th LJk-5C had degraded linuron to 0.32ppm a degradation level of 99.36%. There were significant differences (P<0.05) in the level of linuron degradation between each day over time among the test isolates (Appendix 5). Only isolate LWa-2C that had no significant difference (P> 0.076) in the level of linuron degradation on the 77th and 84th day of incubation.

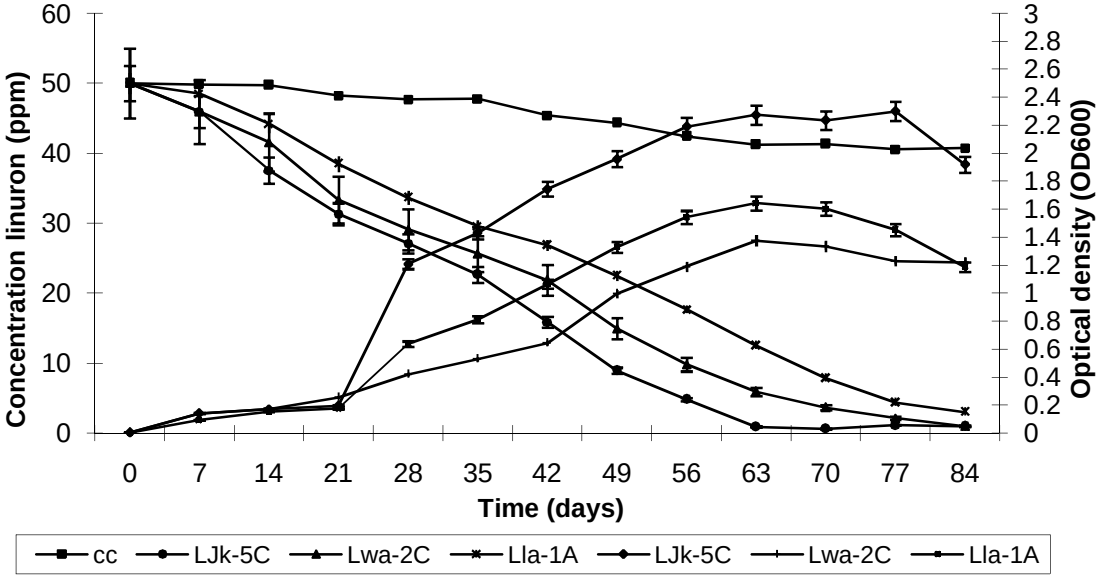

Figure 12a: Degradation of linuron by isolate Ljk-5C, Lwa-2C and Lla-1A. The data points and error bars show the means and standard deviations.

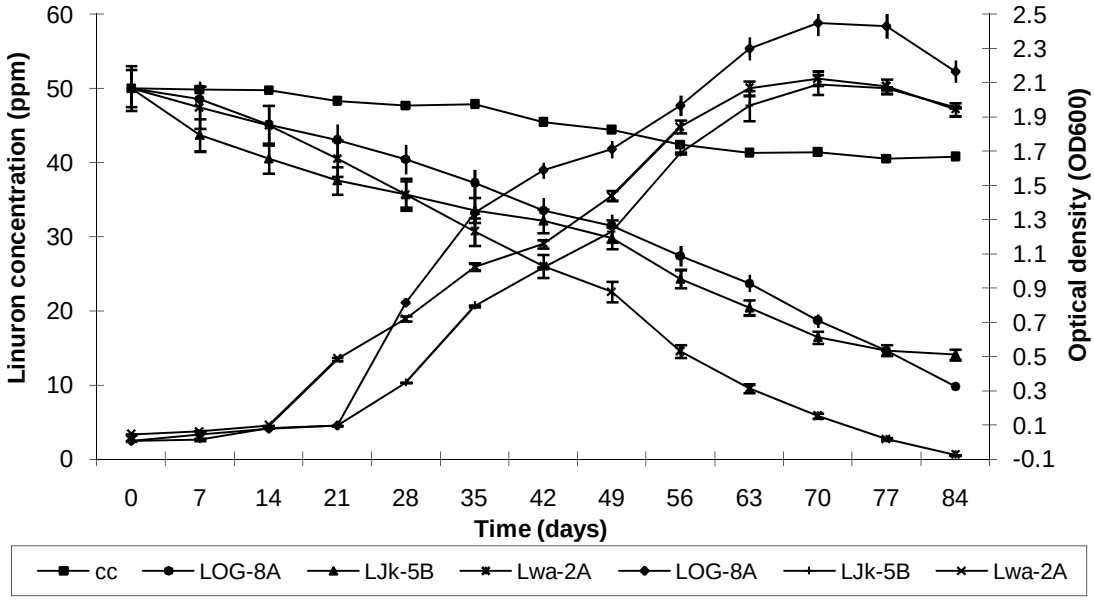

Figure 12b: Degradation of linuron by isolate LOG-8A, Ljk-5B and Lwa-2A. The data points and error bars show the means and standard deviations

4.4.5 GC-MS identification of linuron degradation intermediate metabolites

Spectrums obtained from gas chromatography mass spetrometre indicated that linuron compound was degraded into five (5) intermediate coumpounds (Table 3) in the degdation pathway. Linuron was hydrolyzed at the amide carboxyl linkange to yeild N-methyl-methoxy carbamic acid and 3,4-chloroanalline which is a persistence compound in the environment. N-methyl-methoxy carbamic is unstable and spontaneously degrades to N, O DMHA which is oxidized to $NH^{+}_{4,}$ CO_2 and H_2O.

Table 3: Main intermediate compounds of linuron degradation

Compound name	Retention time (min)	Molecular weight
N,O dimethylhydroxylamine	5.006	61
3-chloroanalline	10.008	127
4-chloroanalline	10.009	127
3,4 dichloroanalline	18.520	161
N-methyl-N-methxy-carbamic acid.	24.580	232

4.5 Characterization of the isolates

4.5.1 Morphological and cellular characteristics

Colonies of diazinon isolates DGa-2A, DJk-4A, were transparent, round and smooth colonies in shape with a bluish green pigmentation the isolates were gram positive and rod in shape. Isolates DKa-5C, DLla-1A, DJk-4C, DJk-4C, DGa-2B colonies were

translucent, entire, white, gram positive and cocci shapes. DLa-1B, DSH-3A, DKi-6B were transparent, round, smooth and pinpointed colonies, with yellowish pigmentation and rod shaped and a gram positive (Table 4) isolates DKa-5A, DKa-5B, DSh-3C, DWa-7B had a flat appearance irregular transparent smooth and cream in pigmentation, gram negative and rods in shape. Isolate DLOG-8A was smooth, flat, bright orange in appearance, small rods in shape and a gram negative. Isolates DGa-2C, DKi-6C were cocci in shape, gram positive, yellowish/whitish in color, smooth circular and pin pointed.

Linuron degrading isolate (Table 5) had varying morphological characteristics which presumptively grouped them into 8 groups. Isolates LOG-8A was opaque, dull white with irregular colonies, a gram positive rod. Isolates LJk-5A, LGA-4B LSh-6C, and LJk-5C were translucent bluish green pigmented undulated colonies, gram negative rods. Isolates LSh-6A, LKi-3A LSh-6B were translucent pale yellow raised colonies, gram negative rods.

Table 4: Biochemical and morphological characterization of diazinon degrading bacteria

Isolates	DGa-2A, DJk-4A, DWA-7A	DKa-5C, DLla-1A,DJk-4C, DJk-4C,DGa-2B	DLa-1B, DSH-3A, DKi-6B	DKa-5A, DKa-5B DSh-3C, DWa7B	DLOG-8A	DGa-2C, DKi-6C
Morphology						
Gram stain	-	-	+	-	-	+
Motility	-	+	-	+	-	-
Shape	Rods	coccobacillus	Rods	Rods	Small rods	cocci
Colony color	Orange	White	yellowish	cream	Bright orange	Golden yellow/whitish
Biochemical						
Catalase	+	+	+	+	+	+
Urease	-	-	+	+	-	+
Gelatinase	-	-	+	-	-	-
Oxidase	-	-	-	+	+	-
Indole	-	-	-	+	-	-
Nitrate reduction	+	+	+	+	+	-
OF	NA	F	F	F	NA	NA
MR	+	+	+	+	+	+
VP	-	-	_	+	-	+

Key: (+) Positive, (-): Negative, MR: Methyl Red, VP Voges-Proskeaur, OF: Oxidation Fermenation

4.5.2 Biochemical characterization of diazinon and linuron degrading isolates

Gram test on diazinon degrading isolates revealed that 48% of the bacteria isolated were in the Gram negative. Motility test done on SIM medium showed that some of the bacteria were motile with the presence of flagella DKa-5C, DLla-1A,DJk-4C, DJk-4C,DGa-2B, DGa-2C, DKi-6C were positive for motility (Table 4). The ability of isolates to excrete intracellular enzymes was determined through tests on fermentation, nitrate reduction, catalase reaction urease, ethyl red, voges-proskauer. All diazinon degrading isolate were positive for nitrate reduction test since the addition of sulfanilic acid and alpha- naphthylamine reacted with nitrite released from nitrate and turn red in color, except isolates DGa-2C, DKi-6C. All isolates were positive for methyl-red test indicating that they fermented glucose and produced a lot of mixed acids as end product. OF test was carried out to examine the ability of isolates to ferment glucose aerobically carbohydrate metabolism which was characterized as a color change of the medium from blue to yellow. Isolates DKa-5C, DLla-1A, DJk-4C, DJk-4C, DGa-2B, DLa-1B, DSH-3A, DKi-6B, DKa-5A, DKa-5B DSh-3C, DWa-7B were positive for the test. Isolates DLa-1B, DSH-3A, DKi-6B, DKa-5A, DKa-5B DSh-3C, DWa-7B, DGa-2C, DKi-6C were positive for urease test. Catalase test revealed that only isolate LWA-2A was negative for hydrogen peroxide production as end product of oxidation of sugars among the linuron degrading bacteria. Test for urease production showed that out of the eight groups for linuron degrading isolates.

Table 5: Biochemical and morphological characterization of linuron degrading bacteria

Isolates	LOG-8A	LJk-5A, LGA-4B, LSh-6C, LJk-5C	LWA-2A	LWA-2C	LJk-5B	LKa-7A	LSh-6A, LKi-3A, LSh-6B	LLa-1A
Morphology								
shape	Rods	Rods	Rods	Rods	Rod	Coccobacillus	Rods	Rods
Gram stain	+	-	+	-	-	-	-	-
motility	+	+	+	+	+	+	+	+
color	white,	bluish/green	green/ black center	Brown	white	White	pale yellow	yellow
Biochemical								
catalase	+	+	+	+	+	+	+	+
urease	-	-	-	-	+	-	+	-
gelatinase	-	-	+	+	-	-	+	-
oxidase	-	+	+	-	+	-	-	-
indole	-	+	+	-	-	-	+	-
Nitrate reduction	-	+	-	+	+	+	+	-
MR	-	-	-	-	-	+	-	-
VP	-	-	+	+	+	-	-	-
TSI	A/A	K/K	A/A	A/A	A/A	A/A	K/K	K/K

Key: (+) Positive, (-): Negative, MR: Methyl Red, VP Voges-Proskeaur, TSI triple sugar iron, A/A; lactose and/or and glucose fermentation, KK No carbohydrate fermentation or hydrogen sulfide production.

Isolates LWA-2A, LJk-5B, LSh-6A, LKi-3A could break down urea to alkaline end products that raised the pH of the medium, causing the phenol red to turn into a pinkish-red color. Isolates LJk-5A, LGA-4B, LSh-6C, LJk-5C, LWA-2A, LSh-6A, LKi-3A (Table 5) were positive for extracellurar gelatinase enzyme. The ability of isolates to produce cytochrome oxidase which oxidises transport molecule cytochrome c while reducing oxygen to form water. Isolates LJk-5A, LGA-4B, LSh-6C, LJk-5C, LSh-6A, LKi-3A and LSh-6C tested positive for production of tryptophanase enzyme which breaks down amino acid tryptophan to form indole, pyruvic acids and ammonia as end products (Table 5). Dentrification test done indicated that isolate LOG-8A, LWA-2C and LLA-1A did not utilize nitrate as an electron acceptor.

4.6 Assay for extracellular esterase enzyme

Enzymatic Index (EI) was expressed by the relationship between the average diameter of the degradation halo and the average diameter of the colony growth (Figure13). Among the 18 Diazinon degrading isolates 15 expressed different levels of enzymatic index. There were significant differences among the isolates studied ($P<0.05$) in the enzymatic index. DGa-2A (1.352), DSh-3C (1.245), DJk-4B (1.058) and DKa-5A (0.809) had significantly the highest enzyme index compared to other isolates. DJk4-C (0.679), DLoG-8A (0.652), DGa-2B (0.591), DLa-1B (0.608), DLa-1A (0.592) and DSh-3A (0.507) had modest level enzyme index of above 0.5 enzymatic level index compared to all isolates. Dki-6B (0.447), Dka-5B (0.414) and DGa-2C (0.3939) expressed enzyme indexes lower than 0.5 units. The highest halo diameters followed Dsh-3A (17.5mm), Dka-5C (16.5mm) and Dwa-7B (13.25mm). DSh-3C (4.5mm), Dki-6B (4.5mm) and Dka-5A (4.5mm) halo diameters were insignificant. Isolate DWa-7B was observed to be the least liberator recording 0.358 units of enzymatic index.

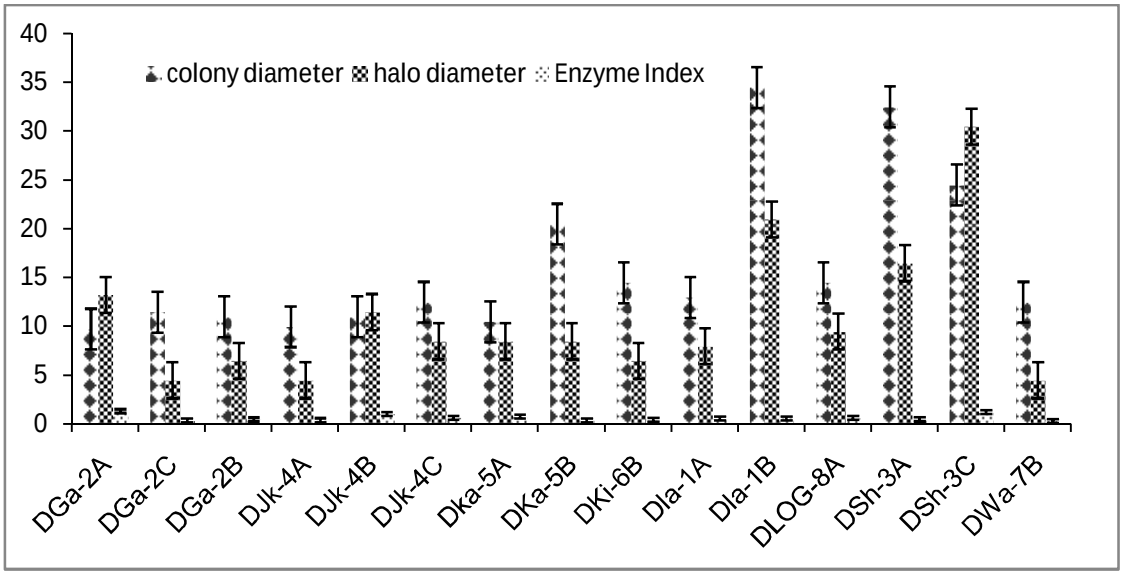

Figure 13: Levels of enzymatic index expressed by diazinon degrading isolate

Among the thirteen Linuron isolates only 5 expressed different levels of enzymatic index (Figure 14). Levels in enzyme index ($P<0.05$) expressed by the isolate were significantly different. Lki-3A (1.747), LJk-5B (1.553) and LSh-6A (1.471) had significantly the highest enzyme indexes.

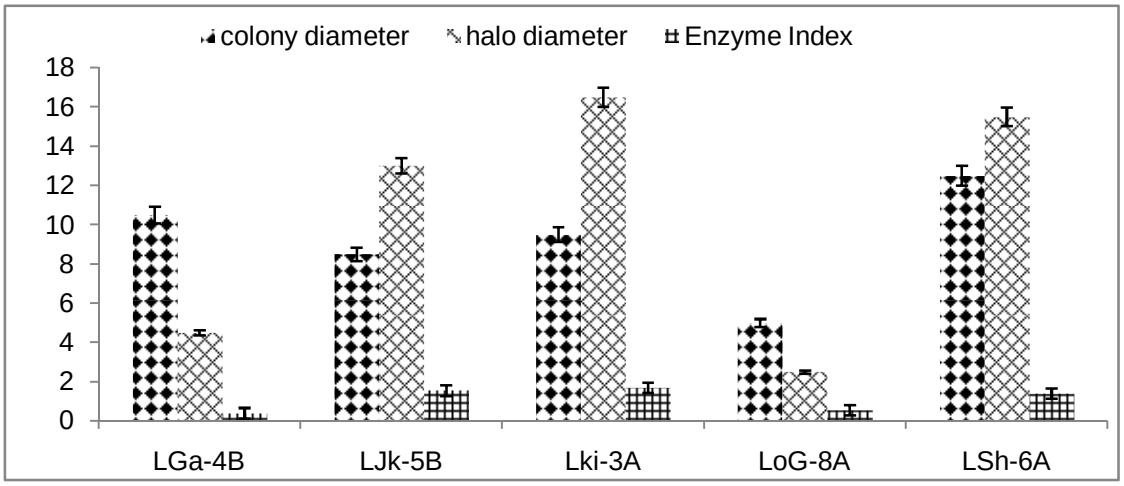

Figure 14: Levels of enzymatic index expressed by linuron degrading isolates

4.7 Assay for alkaline phosphatase

Extracellular alkaline phosphatase activity was observed in all diazinon degrading isolates tested (Table 6). Isolate DWa-7A (EI 0.843) was the only isolate that presented a significant difference at the level of $P < 0.05$ for the test when compared to others.

Table 6: Phosphatase enzymatic activity of diazinon degrading isolates

Isolate	p-nitrophenolate(µg/ml)	Total protein(µg/ml)	Enzymatic index*
Dki-6B	0.24 ± 0.006^{c}	53.47 ± 0.366^{a}	0.004 ± 0.006^{a}
DJk-4A	0.13 ± 0.016^{d}	49.30 ± 0.347^{de}	0.002 ± 0.003^{a}
DLoG-8A	0.36 ± 0.013^{b}	47.59 ± 0.442^{c}	0.008 ± 0.002^{a}
Dwa-7A	0.70 ± 0.028^{a}	47.49 ± 0.217^{c}	0.084 ± 0.004^{b}
Dki-6A	0.13 ± 0.004^{d}	44.92 ± 0.313^{d}	0.003 ± 0.003^{a}
DSh-3A	0.12 ± 0.001^{d}	44.40 ± 0.168^{de}	0.002 ± 0.002^{a}
Dwa-7B	0.13 ± 0.005^{d}	44.37 ± 0.368^{de}	0.001 ± 0.003^{a}
DSh-3B	0.12 ± 0.003^{d}	44.05 ± 0.538^{fg}	0.003 ± 0.001^{a}
Dka-5C	0.40 ± 0.057^{b}	43.49 ± 0.767^{ef}	0.010 ± 0.010^{a}
Dka-5A	0.11 ± 0.009^{d}	42.24 ± 0.586^{gh}	0.003 ± 0.002^{a}
DJk-4B	0.12 ± 0.005^{d}	41.36 ± 0.471^{h}	0.005 ± 0.003^{a}
DGa-2C	0.13 ± 0.0037^{d}	38.14 ± 0.648^{i}	0.003 ± 0.002^{a}
DGa-2A	0.22 ± 0.011^{c}	36.94 ± 0.284^{ij}	0.004 ± 0.006^{a}
Dka-5B	0.24 ± 0.012^{c}	36.92 ± 0.317^{ij}	0.006 ± 0.004^{a}
DGa-2B	0.24 ± 0.013^{c}	36.25 ± 0.501^{j}	0.005 ± 0.001^{a}
D1a-1A	0.12 ± 0.009^{d}	35.95 ± 0.272^{j}	0.005 ± 0.002^{a}
DJk-4C	0.25 ± 0.003^{c}	30.99 ± 0.603^{k}	0.008 ± 0.003^{a}
D1a-1B	0.03 ± 0.020^{e}	5.21 ± 0.063^{l}	0.007 ± 0.005^{a}
CV %	16.47	2.186	16.5
$LSD_{0.05}$	0.0495	1.247	0.04865

***** The enzymatic index represents the ratio of p-nitrophenolate/total protein. Means separated using LSDs' test by the same letter are not significantly different ($P<0.05$) from each other.

Significant differences (P<0.05) were observed in levels of enzymatic index expressed by linuron degraders for phosphatase activity (Table 7). Isolate Lki-3A recorded the highest amount of enzymatic index I.E for extracellular phosphatase activity (0.0064), followed by Lla-1A (0.0056), Lwa-2A (0.0027) and LSH-6B (0.0037). All other isolates had no significant difference (P= 0.1543) in the E.I.

Table 7: Phosphatase enzymatic index of linuron degrading isolates

Isolate	p-nitrophenolate (µg/ml)	Total protein (µg/ml)	Enzymatic index*
Lwa-2A	0.234 ± 0.002^{a}	91.523 ± 0.524^{h}	0.002 ± 0.002^{c}
Lki-3A	0.101 ± 0.001^{b}	147.645 ± 0.786^{c}	0.006 ± 0.006^{f}
Lka-7A	0.073 ± 0.001^{c}	44.843 ± 0.262^{bc}	0.001 ± 0.001^{b}
Lla-1A	0.062 ± 0.001^{d}	99.391 ± 0.262^{h}	0.005 ± 0.005^{e}
LGa-4B	0.036 ± 0.001^{f}	115.388 ± 0.262^{f}	0.000 ± 0.000^{a}
LSh-6B	0.032 ± 0.001^{fg}	94.932 ± 0.262^{h}	0.003 ± 0.003^{d}
LJk-5B	0.031 ± 0.014^{fg}	190.129 ± 2.622^{a}	0.000 ± 0.000^{a}
LSh-6C	0.026 ± 0.003^{gh}	125.007 ± 0.761^{e}	0.002 ± 0.002^{bc}
LSh-6A	0.024 ± 0.0005^{h}	136.892 ± 0.262^{d}	0.000 ± 0.000^{a}
LoG-8A	0.013 ± 0.005^{i}	176.099 ± 0.131^{b}	0.000 ± 0.000^{a}
LJk-5A	0.003 ± 0.0005^{j}	109.881 ± 0.262^{g}	0.000 ± 0.000^{a}
Lwa-2C	0.051 ± 0.003^{c}	108.045 ± 0.262^{g}	0.000 ± 0.000^{a}
LJk-5C	0.017 ± 0.001^{i}	128.500 ± 0.262^{de}	0.000 ± 0.000^{a}
CV%	16.8	0.9	18.00
$LSD_{0.05}$	0.0004	0.6	0.000723

***** The enzymatic index represents the ratio of phenolate/total protein. Means separated using LSDs' test by the same letter are not significantly different (P<0.05) from each other.

The linear relationship between total protein liberated level and phenolate liberation among diazinon degrading isolates was described by R^2 values in each isolate (Table 8).

The low R^2 indicated the two parameters were very slightly interrelated. Isolate Dka-5C had the strongest relationship with a R^2 of 0.9148. Isolate Dwa-7A and Dki-6B had R^2 values of 0.816 and 0.515 respectively an indication that total phenolate liberated was dependent on the amount of total protein expressed by the isolates (Table 8). Isolates that had R^2 values below 0.1 includes DLa-1B (0.0750), DGa-2B (0.0005), DJk-4B (0.0033), DGa-2A (0.0109) and DLoG0-8A (0.0007) a low insignificant positive regressions.

Table 8: Relationship of total protein to p-nitrophenolate liberation by diazinon degrading isolates

Isolates	Equation	R^2
DGa-2A	Y = -0.0041x + 0.3793	0.01
DGa-2B	Y= -0.0006x + 0.2658	0.00
DGa-2C	Y = 0.0002x + 0.1245	0.12
DJk-4A	Y = 0.0122x – 0.4639	0.13
DJk-4B	Y = -0.0052x + 0.3418	0.16
DJk-4C	Y = 0.0003x + 0.2443	0.00
DKi-6A	Y = 0.5155x + 0.5733	0.51
Dki-6B	Y = 0.0086x – 0.2162	0.22
Dka-5A	Y = 0.015x -0.5474	0.91
Dka-5B	Y = -0.0174x + 0.8876	0.19
Dka- 5C	Y = 0.035x -1.1197	0.21
Dla-1A	Y = 0.019x -0.5661	0.30
Dla-1B	Y = 0.091x -0.4402	0.07
DloG- 8A	Y = -0.0007x +0.4003	0.00
DSh-3A	Y = -0.0125x + 0.6843	0.25
DSh-3C	Y = -0.0027x + 0.2445	0.21
DWa-7A	Y = 0.1177x -4.8792	0.81
DWa -7B	Y = 0.0057x – 0.12	0.12

Comparison of the relationship between total protein and phenolate liberation by linuron degrading bacteria (Table9) revealed that isolate Lka-7A had the strongest of total

protein to phenolate liberation with R^2 value of (0.904) followed by LJk-5A (0.868), LKi-3A (0.809) and LGa-4B (0.797) a regressions of (r^2 >0.7). It was observed that isolates LoG-8A (0.114), LSh-6A (0.331), LSh-6C (0.041).

Table 9: Relationship of total protein to p-nitrophenolate liberation by linuron isolates.

Isolates	Equation	R^2
LGa-4B	y=-0.0011x+0.1583	0.79
LJk-5A	y=0.0001x-0.0066	0.86
LJk-5B	y=0.0001x+0.0042	0.11
LJk-5C	y=0.0003x-0.0219	0.50
Lka-7A	y=0.0034x-0.0783	0.90
Lki-3A	y=-0.0006x+0.1946	0.80
Lla-1A	y=0.0016x-0.0964	0.68
LoG-8A	$y=5.2310^{-7}x+0.0047$	0.11
LSh-6A	$y=5.6310^{-7}x+0.0163$	0.03
LSh-6B	y=0.0005x-0.0123	0.50
LSh-6C	y=0.0003x-0.0137	0.04
Lwa-2A	y=-0.0442x+4.1945	0.59
Lwa-3C	y=-0.034x+3.7272	0.73

4.8 Molecular characterization of isolates

PCR products of the 16S rRNA of the linuron degrading isolates with a molecular weight of 1500 base pairs (bp).

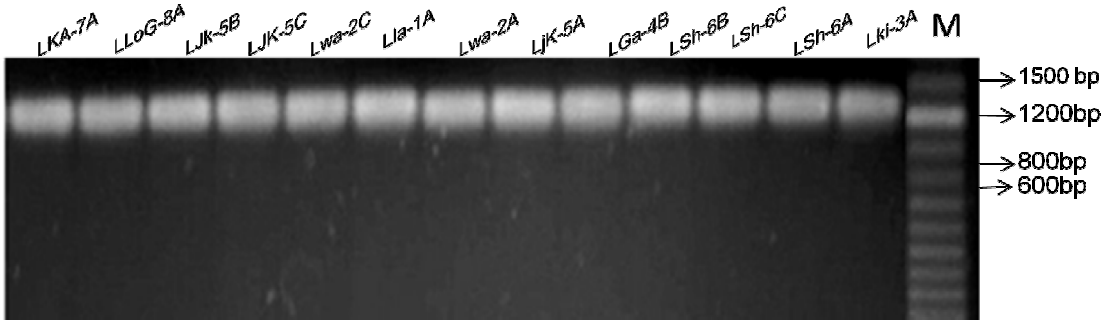

Figure 15: PCR products of linuron degrading bacteria

The restriction enzyme analysis provided different clustering patterns of the bacteria which divided them into four groups namely; A, B, C and D.

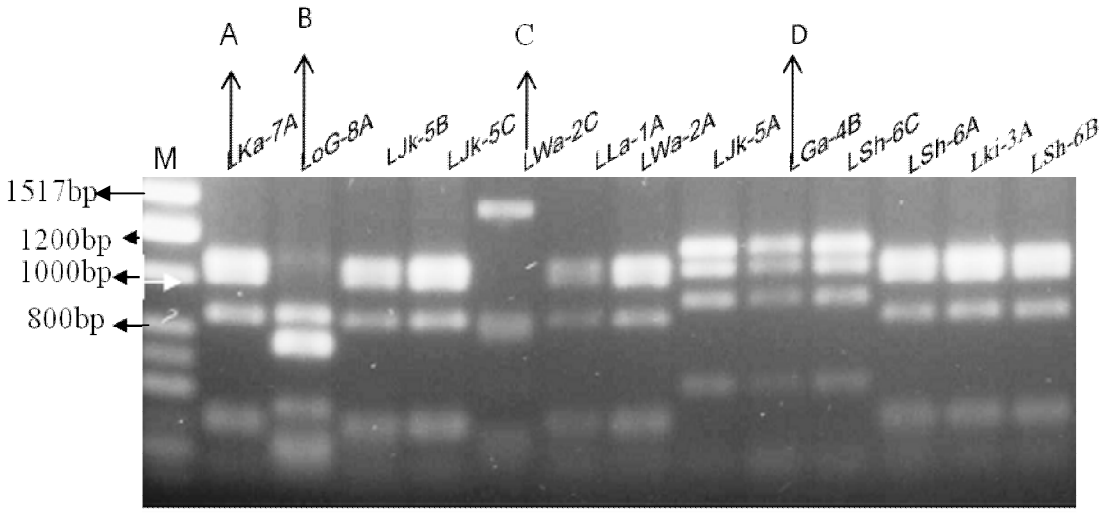

Figure 16: PCR products restriction profiles of Linuron degrading bacteria

4.9 Phylogenetic analysis of bacterial isolates

Isolate DGa-2C, Dla-1A, DKa-5C, DJk-4C, DGa-2B, Dki-6A and DKa-5C were closely related to *Enterobacter* sp, with a sequence similarity of 95%-99%. Isolates Dla-1A and DGa-2B could be novel isolates since their sequence similarities with the data base sequences is < 97-%. DGa-2A, DJk-4A and DWa-7A showed 97-% similarity with *Pseudomonas sp* (Table 11). Strains that clustered closely with *Klebsiella* sp were DSh-3A, DKi-6B and DSh-3A at 99-% sequence similarity. Isolate DLoG-8A showed a 95-% sequence similarity with *Paracoccus sp* from NCBI data gene bank. Dki-6A and DGa-2C clustered with *Staphylococcus* sp with 97-% sequence similarity (Table 11). Isolate DSh-3C had a 98-% sequence similarity with Proteus mirabilis DWa-7B had 97-% similarity with *Proteus sp,* however isolate Dka-5A had <96-% sequence similarity with *proteus* sp. Phylogenetic tree of linuron biodegrading isolates (Figure 18) constructed based on 16s rRNA gene NCBI libraries revealed in this study that 13 linuron degrading bacterium clustered into 8 genera namely; *Pseudomonas* sp, *Xathomonas* sp, *Stenotrophomonas* sp, *Bacillus* sp, *Enterobacter* sp, *Arthrobacter* sp *Myroides* sp and *Burkholderia* sp. Strain LSh-6C, had > 97-% sequence similarity with *Pseudomonas sp.* LLoG-8A, had 98 % similarity with *Bacillus sp*(Table 12). LKa-7A had 97-% similarity with *Enterobacter sp.* LLa-1A had 97-% similarity with *Myroides* sp. LWa-2C had 99-% sequence similarity with *Burkholderia sp* (Table 12). Isolate LJk-5B had a 98-% sequence similarity with *Xanthomonas sp*. Isolates LJk-5C, LJk-5A and LGA-2B had < 97-% sequence similarity with NCBI databse sequences of *pseudomonas* sp.

Table 10: 16S rRNA gene sequence similarity of diazinon isolates and close relatives

Isolate	Accession Number	Next neighbour in BLAST	% Identity
DLoG-8A	AJ580352	*Paracoccus sp.*	95
	NR025857	*Paracoccus aminovorans*	94
	NR024658	*Paracoccus carotinifaciens*	93
DWa-7B	GU477712	*Proteus mirabilis*	96
	AM232728	*Proteus sp*	96
	FJ753846	*Swine fecal bacterium*	95
DGa-2B	FJ745300	*Enterobacter sp*	95
	AB461798	*Enterobacteriaceae bacterium*	95
	HM218110	*Enterobacter homaechei*	98
DKi-6A	GQ222241	*Staphylococcus sp.*	97
	GQ222240	*Staphylococcus xylosus strain*	97
	HM113469	*Staphylococcus saprophyticus*	97
DKa-5C	HM355728	*Enterobacter hormaechei*	99
DJK-4C	HM355727	*Enterobacter asburiae*	99
DKa-5B	NR025336	*Proteus vulgaris*	97
DSh-3C	DQ364577	*Proteus penneri*	97
	DQ885262	*Proteus hauseri*	96
DKa-5A	EU710747	*Proteus sp.*	96
	EF012713	*Proteus vulgaris*	95
DLa-1A	GU086162	*Enterobacter sp.*	97
	HM217949	*Enterobacter cloacae*	97
DWa-7A	GU325751	*Pseudoalteromonas*	97
DJK-4A	AB571944	*flavipulchra*	97
	FJ457179	*Pseudoalteromonas sp.*	97
DGa-2A	GQ330905	*Pseudoalteromonas piscicida*	97
	FJ457168	*Pseudomonas sp*	97
	GQ330905	*Pseudoalteromonas rubra*	97
		Pseudomonas putida	
DLa-1B	AY292872	*Klebsiella sp.*	99
DKi-6B	AF129444	*Klebsiella trevisanii*	99
DSh-3A	GQ259887	*Klebsiella pneumoniae*	99

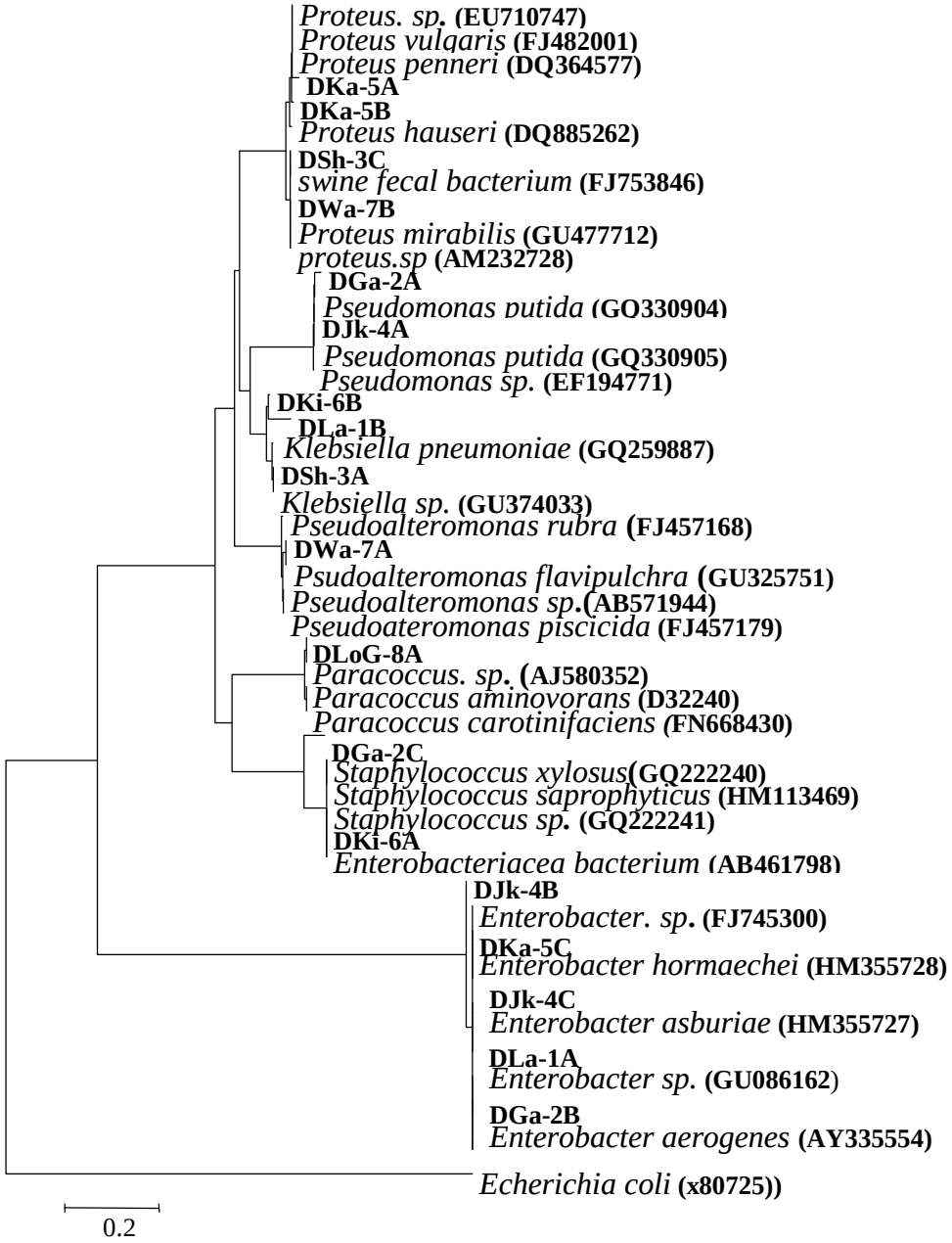

Figure 17: Phylogenetic relationship of diazinon degrading bacteria. The scale bar indicates approximately 2% sequence difference. The evolutionary history was inferred the Neighbor-joining method. Phylogenetic analyses were conducted in MEGA4.

Table 11: 16S rRNA gene sequence similarity of Linuron isolates to other Close relatives

Isolate code	Accession Number	Next neighbour in BLAST	% Identity
LWa-2A	AY641537	*Arthrobacter sp*	97
	GQ921838	*Arthrobacter sp*	97
	EU086809	*Arthrobacter aurescens*	97
	FN908790	*Arthrobacter globiformis*	96
LKi-3A	EU434429	*Stenotrophomonas sp*	98
	AM403589	*Stenotrophomonas terrae*	97
LSh-6B	AB294556	*Stenotrophomonas maltophilia*	97
LSh-6A	AM403587	*Stenotrophomonas humi*	95
	FJ482062	*Stenotrophomonas nitritireduns*	95
LWa-2C	GQ468397	*Burkholderia sp*	99
	AY512825	*Burkholderia cepacia*	99
	GU433447	*Burkholderia cenocepacia*	99
LLa-1A	GQ383900	*Myoides odoratimimus*	97
	M58777	*Myroides ordoratum*	97
	GU350455	*Myroides sp*	97
LJk-5B	*FN645733*	*Xanthomonas sp*	98
	AF209754	*Xanthomonas bromi*	98
LOG-8A	*AM910270*	*Lysinibacillus sp*	99
	GU204967	*Lysinibacillus sphaericus*	98
	FJ418643	*Lysinibacillus fusiformis*	98
LKi-3B	FJ588708	*Enterobacter sp*	97
LKa-7A	AM943033	*Enterobacter hormaechei*	97
	AB244288	*Enterobacter cloacae*	97
	AF395913	*Enterobacter aerogenes*	95
	HM161855	*Enterobacter asburiae*	95
LSh-6C	FJ950542	*Pseudomonas fluorescens*	97
	DQ377758	*Pseudomonas miquale*	96
LJk-5C	HQ236540	*Pseudomonas monteilli*	96
LGa-4B	FJ950654	*Pseudomonas pituda*	95
	AM410620	*Pseudomonas fulva*	96
Root	X80725	*Echerichia coli*	
LJK-5A	DQ095915	*Pseudomonas plecoglossicida*	96
	EU103629	*Pseudomonas taiwanesis*	96

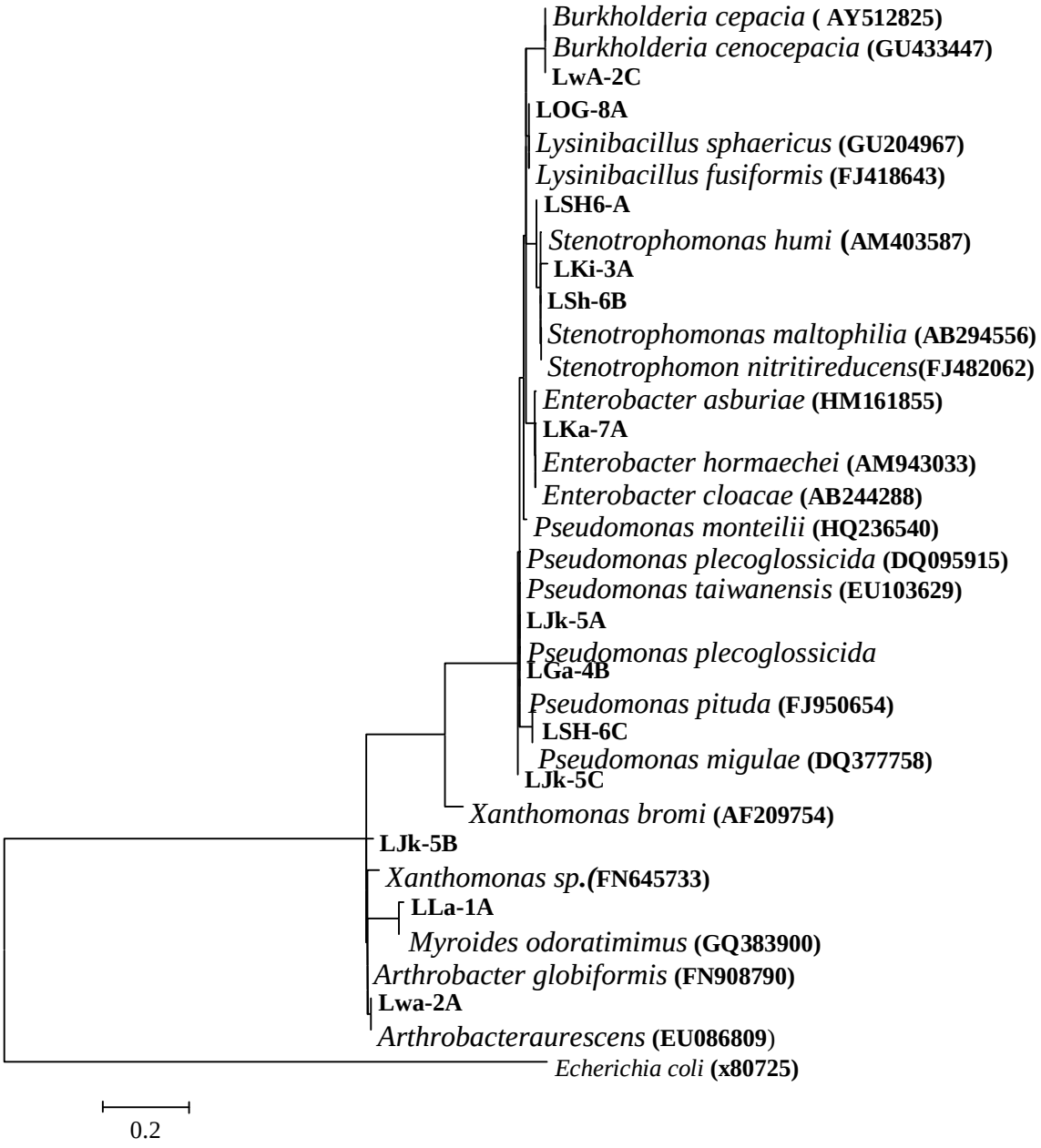

Figure 18: Phylogenetic relationship of the linuron degrading bacteria. The evolutionary historywas inferred using the Neighbor-Joining method. Phylogenetic analyses were conducted in MEGA4**.**

CHAPTER FIVE

5.0 Discussion, conclusions and recommendations

5.1 Discussion

5.1.1 Pesticide use in horticultural farms

Survey results revealed that types of pesticides used by the farmers in the study areas were mainly insecticides (44%), herbicides (34%) and fungicides 13% with the remaining 9% being nematocides. These results are in line with Epstein and Bassein, (2003) who observed that farmers in Tanzania used more insecticides (59%) because they based the applications on calendar spray programs without necessarily giving much priority to health and environmental considerations. The study also revealed that of the different insecticide and herbicides formulation used by farmers most insecticides were in the chemical class of organophosphates. The results confirms earlier reports by (Poorva *et al.,* 2010), that among the various groups of pesticides being used all over the world, organophosphorus pesticides form a major and most widely used group accounting for more than 36% of total world market. Phenyl urea herbicides were second in use with a 12% usage level. Reports by Rana, 2010 showed a 45% relative increase in use of herbicides when compared to fungicides and insecticides between the year 2003 and 2009 in the world.

5.1.2 Diazinon and Linuron degrading bacteria from soils under horticultural production.

Linuron and diazinon degrading bacteria were screened, including those potentially responsible for partial transformation. Aliquots of soil enrichment cultures were obtained at the stage of rapid linuron and diazinon degradation 42 and 18 days after treatment respectively; using the conventional pour plate method. The aliquot were serially diluted with sterile minimum mineral salts medium (MSM) and inoculated into plates containing minimum mineral salts in 2 % agar supplemented with 50 and 12 mg L^{-1} linuron and diazinon. The plates were then incubated for 14 days at 28^0C. Thirteen and eighteen colonies were randomly picked from the agar plates with a $10^{-3.}$ The isolates were sub cultured and pure cultures obtained.

5.1.3 Cultural and biochemical characteristics of pesticide degrading bacteria

Diazinon degrading isolates DGa-2A, DJk-4A, DWa-7A, Dka-5C, DLla-1A, Djk-4C, DGa-2B, DLla-1B, DSh-3A, DKi-6B, DKa-5A, DKa-5B, DSh-3C, DWa-7B and DLoG-8A were gram negative, rods in shape. Linuron degrading isolates were all gram negative except for LoG-8A and Lwa-2A. These results are in line with those of Alexander and Strete 2001 that gram negative bacteria have a thin peptidoglycan and an additional lipopolysaccharides layer hence lose the primary stain crystal violet during decolorization with 95% ethanol and took up the counter stain safranin. Isolates DGa-2C, DKi-6C and LoG-8A, Lwa-2A were gram positive bacteria and did not take up the counter stain after decolorization with 95% ethanol. Microscopy examination of the

isolates after staining them with a basic stain crystal violet identified them as either rods or coccus in shape. Diazinon degrading isolates DKa-5C, DLla-1A, Djk-4C and DGa-2B and linuron degrading isolates were identified as coccus. This observation conformed with the work reported by Alexander and Strete 2001 that morphological stains on the basis of the charge they carry can either be attracted to the bacterial cell if they carry opposite charge and color the bacterial cells or the can be repelled by the cells if they carry the like charges hence coloring the background surrounding enabling the outline of the bacterial cell to be observed.

Motility test carried out on the isolates degrading both pesticides to determine the present of flagella showed that majority of the bacteria that degraded diazinon did not have the ability to spread away from the line of inoculation only isolate DKa-5C, DLla-1A, DJk-4C and DGa-2B tested positive for motility. Linuron degrading isolate were all positive for the test.

Diazinon and linuron isolates were all positive for catalase test. Catalase test detects the catalase enzyme present in most cytochrome-containing aerobic bacteria which form Hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars, decomposing hydrogen peroxide to water and oxygen as earlier described by (Cappuccino and Sherman, 2002). The ability of the isolates to attack amide bonds in the pesticides was determined using urea broth medium. Diazinon degrading isolates Dla-1B, DSh-3A, DKa-5A, DKa-5B, DSh-3C, Dwa-7B, DGa-2C and DKi-6C and linuron degrading bacteria LWa-2A, LJk-4B, LSh-6A and LKi-3A were positive for Urease test.

Splitting of urea compound by the bacteria resulted in formation of two units of ammonia which raised the pH in the medium from 6.8 to 8.4 hence turning the phenol red indicator to rose pink conforming to previous report by (Harold, 2002).

Nutrient broth supplemented with 12% gelatin was used to demonstrate hydrolytic activity of gelatinase enzyme. Diazinon degrading isolates DLla-1A, DSh-3A and DKi-6B and linuron degrading bacteria LWa-2A, LSh-6A and LKi-3A showed positive results. After incubation the cultures remained liquefied when placed in refrigerator at 4^0c for 30 minutes as earlier indicated by Cappuccino and Sherman, 2002. In oxic environment some bacteria use oxygen molecule as the final electron as in an effort to respire and obtain energy. The final reaction in the electron transport chain is catalyzed by the cytochrome oxidase where cytochrome oxidase oxidizes the electron transport molecule cytochrome c, while reducing oxygen to form water. Diazinon degrading bacteria DKa-5A, DKa-5B, DSh-3C, Dwa-7B and DLoG-8A and linuron degraders LJk-5A, LGa-4B, Lsh-6C, Ljk-5C, LWa-2A and LJk-5B were found to be cytochrome oxidase positive since they had the ability to oxidize the test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor impregnated in a test strip which upon transferring growth 24 hr old culture colonies there was a color change to blue as described by (Cappuccino and Sherman, 2002).

The ability of the isolates to reduce nitrates to nitrites or beyond by liberation of nitrate reductase was carried out using nitrate reduction broth. All diazinon isolates tested positive except for DGa-2C and DKi-6C while in linuron degraders Lla-1A and LWa-2C

had positive results. Following inoculation and incubation, the addition of sulfanilic acid and alpha- naphthylamine these two compounds reacted with nitrite and turn red in color, indicating a positive nitrate reduction test as described by (Alexander and Strete 2001).

Indole generation by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid was tested for diazinon and linuron degrading bacteria. Isolates DKa-5A, DKa-5B, DSh-3C, DWa-7B while linuron isolates LJk-5A, LGa-4B, Lsh-6C, Ljk-5C, LWa-2A, Lsh-6A, Lki3A and Lsh-6B were positive for the test. Tryptophanase enzyme catalyzes the deamination reaction, during which the amine (-NH_2) group of the tryptophan molecule was removed and final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy as previously described by Harold, 2002.

Hydrolytic enzymes catalyze the clevage of certain chemical bonds of a substrate the by addition of the components of water (H or OH) to each of the products. A wide array of hydrolases (amidies, esrerases, Iipases, nitrilases, peptidases, phosphatases, etc.), with broad and narrow substrate specificities are present in animals, microorganisms (Rahmansyah *et al.,* 2009). Isolates production of alkaline phosphatase was assessed by their ability to reduce 1mM of p-nitrophenyl phosphate to p-nitrophenolate per minute. All diazinon degrading isolates expressed different levels of the phosphatase activity; DWa-7A had the highest activity liberating 0.7 ug/ml of p-nitrophenolate per minutes while DLla-1A had the least activity with 0.03 ug/ml of p-nitrophenolate per minute.

Linuron degraders showed very low activity of phosphatase liberation, isolate LWa-2A had the highest with 0.2 ug/ml of p-nitrophenolate while LJk-5A had the least phosphatase activity yielding 0.003 ug/ml of p-nitrophenolate per minute. Alkaline phosphatase is a broad term associated with non-specific phosphomonoesterase activity with activity optima at alkaline pH. It is a homodimeric metalloenzyme that catalyzes the cleavage of a phosphate group from a variety of compounds into inorganic phosphate and corresponding alcohol (Robert and Evan 2003). Organic and inorganic phosphates are essential component of living organism, Oxidation processes of the enzymes often makes the pesticide more amenable to degradation at the extracellular level and then proceed further at the intracellular level that causing increase its water solubility, and lastly increasing bioavailability as earlier reported by (Rahmansyah *et al.,* 2009).

Esterase activity of the isolate was estimated by their ability to precipitate calcium salts in the culture medium using Tween 80 as the substrate and form clearing zones around the bacterial colony. Diazinon degrading isolates showed a wide range of enzymatic index ranging between 1.35mm - 0.35 units. Isolate DGa-2A had the highest level of enzymatic index and the least was Dwa-7B with an enzymatic index of 0.358 units. In linuron degraders isolate LKi-3A had the highest level of enzymatic index with 1.7 units and LoG-8A was the least with 0.55 units of esterase. In a related pesticide biodegradation study by Golovleva *et al*., 1990 reported that due to bacteria hdrolases amide and ester bonds underwent hydrolytic cleavage in anilides, phenylureas, esters of carbamic, thiocarbamic, phosphoric, thiophosphoric, and of other acids. In the study bacteria isolates that had high levels of hydrolytic enzymes were not observed to be the

best biodegraders of the study compounds' this phenomenon was attributed to earlier reports by Singh and Walker 2006 that the way in which metabolism is regulated in a bacteria depends very strongly on what role the compound plays for the particular organisms and the relevant gene cannot be expressed as a response to starvation for another of these elements.

5.1.4 Molecular characterization of isolates and degradation level of diazinon and linuron by soil bacteria.

Amplification of the 16S rRNA gene allowed generation of high copy numbers of these genes, which were subsequently used for bacterial identification. Cleavage of PCR-generated 16S rRNA gene amplicons by a restriction enzymes resulted in differentiation of the isolate into different group. The 16S rRNA PCR-RFLP were separated by electrophoresis and visualized on an agarose gel which revealed that linuron degrading bacteria had four groups and diazinon degrading isolates had 13 groups. Definitive identification of the isolates was achieved by comparing the sequenced 16S rRNA genes with those available in the NCBI databases. The 16S rRNA PCR-RFLP of the study did not match with those of NCBI database. BLAST result revealed that linuron and diazinon degrading bacteria had 13 and 18 organisms rather than the 4 and 13 groups differentiation revealed by PCR- RFLP. This observation could be attributed to occurrence of dimorphic site within a singe genome hence this method allows only for the identification of group related species rather than defined species. These observations are in line with those of (Oger *et al.,* 1998) who observed that bacteria belonging to the same

genospecies of *Xanthomonas* give indentical 16S rRNA patterns except for strain LMG 852 which differed from other members by one restriction site.

Bacterial isolates were inoculated into Erlenmeyer flask containing MMS medium with linuron 50 mg L−1 and 12 mg L−1 of linuron and diazinon. After incubation period of 7 and 5 days in the dark at 28^0C, 10 µL aliquots of the culture medium from each tube were HPLC analyzed. Diazinon degraders DGa-2B, DJk-4B, DKA-5C, DLLa-1a and DJK-4C had a removal capacity of diazinon of between 40-76 % at the end of a 35 day incubation period. Sequence analysis of the isolates revealed that they clustered with members of the genus *Enterobacter*. Phylogenetic position of the 5 isolates showed they clustered more closely with *Enterobacter hormaechei* with 98% sequence similarity. This was supported by high bootstrap values of 50-99%. *Enterobacter hormaechei* has both a respiratory and a fermentative type of metabolism. D-Glucose and other carbohydrates are catabolized with the production of acid and gas and also been shown be N_2-fixers in soil (Krieg, *et al.*, 1993). In a similar organophosphorus pesticide degradation studies *Enterobacter* B-14 isolated from Australian soil has been reported to degrade chlorpyrifos a compound used as an alternative to diazinon in control of pests in crops. In MSMN without any other source of carbon, all chlorpyrifos was degraded in 2 days (Brajesh *et al.*, 2003).

DNA sequences of DLoG-8A and phylogenetic tree result based on representatives of validly described the isolate as a *Paracoccus* species. The sequence of DLOG-8A was 93–95% similar to the sequences of *P. aminovorans* strain (GenBank accession no.

NR025857), *P. carotinifaciens* strain (GenBank accession no NR024658) (Lipski *et al.*, 1998; Rainey *et al.*, 1999). These biochemical characteristics together with Phylogenetic analysis confirmed the biochemical tests and placed isolate DLOG-8A as a member of the genus *Paracoccus*. The isolate was capable of 76% of diazinon removal within a culture period of 35 days. In a related pesticide biodegradation study in China members of the genus *Paracoccus* have been reported to degrade several organophosphorus compounds like monocrotophos 500 mg L^{-1} with a removal percentage of 79.92% within 6 h of culture in liquid mineral salt medium (Kai-zhi *et al.*, 2006). This shows that the strain is more efficient in monocrotophos removal compared to diazinon.

Phylogenetic analysis of 16S rRNA gene sequence of isolates DKa-5A, DKa-5B and DWa-7B confirmed the morphological and biochemical characteristics of the isolates and clustered them with members of the genus *Proteus* (Auwaerter *et al* 2008). *Proteus sp* belongs to the family of Enterobacteriaceae, members of the genus *Proteus* occur widely in man, animals and in the environment and can be readily recovered from sewage, soil, garden vegetables and many other materials (Hickman *et al.*, 1982). In a related study in Mexico, *Proteus vulgaris* was reported to have a 14.6% removal percentage of tetrachlorovinphos from minimal medium within 72 h while in this study the bacterium had a 8.4% removal of diazinon from the liquid culture medium within the same culture period. Tetrachlorvinphos share a phosphoric acid derivative chemical structure as diazinon.

In this study isolates DGa-2A and DJk-4A clustered closely with *Pseudomonas putida*. The strains had a lag phase of 5 days and degrading 0.5% per day of the initial dose of 12 mg L^{-1}. The results conformed to the work reported by Muriusz *et al.,* 2009 on diazinon degradation where the bacterium had a lag phase of 7 days in which 2% of the initial dose of 50 mg L^{-1} was degraded. The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. Members of the genus are found in large numbers in all of the major natural environments (terrestrial, freshwater and marine) and also form intimate associations with plants and animals. This universal distribution suggests a remarkable degree of physiological and genetic adaptability. The remarkable capacity of *Pseudomonas* strains to degrade a wide range of substrates including aromatic compounds, halogenated derivatives and recalcitrant organic residues has been demonstrated by a study by (Hamzah *et al.,* 1994).

Sequencing the 16 S rRNA genes of the strains DKi-6B, DLLa-1B and DSh-3A and comparing them with previously published 16S rRNA gene sequences, the strains were classified as members of the genus *Klebsiella.* Sequences displayed the highest identity (99%) with the 16S rRNA gene of a *Klebsiella sp*. strain (NCBI database, Gu: 374033). Based on morphological and biochemical characteristics observations, Phylogenetic analysis established the strains as *Klebsiella sp*. The bacterium had the ability to degrade between 53% - 79% of diazinon over 35 days. The fastest degrader was isolate DSh-3A with a removal level of 0.27 ppm per day. In a related study Ghanem *et al.,* 2007 reported a *Klebsiella sp* with the ability to degrade an insecticide chlorpyrifos at a

removal level of 1.5 ppm per day. Chrorpyrifos is characterized by P-O-C linkages as in other organophosphorus pesticides such as diazinon and parathion.

Isolate Dki-6A and DGa-2C clustered with a *Staphylococcus sp.* They had a sequence identity of 97%. The isolates had different diazinon removal percentages. Isolate DKi-6A maintained a consistence trend of degradation and at the end of the culture period 94% 0f the compound was degraded. Isolate DGa-2C had a fastest degradation rate on the first to weeks of incubation but the trend seemed to decline as degradation metabolite accumulated in the culture medium. In a related study (Michel *et al.,* 1996) reported an accumulation of a high molecular weight diazinon degradation residues which are presumed to have low bioavailability for further microbial degradation. A pervious study (Schneewind *et al.,* 1995) reported that a *Staphylococcus aureus* had the potential to express (SpA) protein on it cell membrane. After sequential cycles of DNA shuffling and screening to "fine tune" and enhance the activity of organophosphorus hydrolyase towards poorly degraded substrates. The result was a single microorganism that was endowed with the capability to rapidly degrade organophosphate pesticides and PNP (Shimazu *et al.,* 2001).

Degradation of linuron was monitored over a period of 84 days. All the isolates had a long lag phase of 7 days. An indication that an acclimation process such as induction or depression of enzymes, mutation or genetic exchange, multiplication of the initially small populations of degrading organisms, preferential utilization of other organic

compounds before the chemical of interest, or adaptation to the toxins or inhibitors present as earlier reported by (Cabrera and Lebeault, 1993).

Isolates LSh-6C, LKi-3A and LSh-6B clustered with genus *Stenotrophomonas.* The genus Phylogenetic position of the three isolates showed that they clustered more closely to with *Stenotrophomonas maltophila. S. maltophila* Strain can grow aerobically from 4 °C to 37 °C, but not at 41 °C. Most of the sugars such as glucose, sucrose, fructose, mannose and maltose, and organic acids such as citrate acetate, malate, succinate, lysine, histidine and leucine, can support the growth of strain (Yang *et al.,* 2006). These isolates could degrade linuron at percentages between 10% and 12% within the first 7 days of incubation with accumulation of 3, 4 DCA in the medium as the final degradation products. The findings in this experiment are in line with those of (El-Fantroussi *et al.,* 2000) who observed that *Stenotrophomonas sp* could degrade linuron with an accumulation of 3, 4 DCA in the medium. However the findings in the study are also converse to those of Batisson *et al.,* 2007 who reported a strain designated IB78 isolated from diuron-contaminated lotic surface water and identified as a *Stenotrophomonas sp* had the ability to degrade 3-4 DCA a main metabolite in the degradation pathway of phenyl urea herbicides at a 20% removal percentage within 24 h of incubation.

Phylogenetic analysis clustered isolate LOG-8A with *Lysinibacillus sp.* The strains are ubiquitous in nature: soil, water, and airborne dust (Shareefdeen *et al.,* 2005). Isolate LOG-8A showed 80.32% reduction in concentration of linuron by the 84th day of incubation. This observation agrees with the work of (Cullington *et al.,* 1999) that

isolated *Bacillus sphaericus* ATCC 12123 could use the alkyl chain of linuron as the sole N and C source. However, he also observed that these pure strain could only partially degrade linuron, since 3,4-dichloroaniline (3,4-DCA), one of the main potential metabolites in the degradation pathway of linuron accumulated in the medium and becomes toxic to the linuron degrading bacteria.

Sequence analysis of isolate LLa-1A showed that the isolate belonged to genera *Myroides.* The genus *Myroides* was established by the reclassification of *Flavobacterium odoratum* as *Myroides odoratus*, the type species, on the basis of genomic, chemotaxonomic and phenotypic studies (Vancanneyt *et al.*, 1996). Biochemical and cellular characteristics of isolate LLa-1A were confirmed by sequencing the 16 S rRNA genes of the strains and comparing them with previously published 16S rRNA gene sequences. The sequences displayed the highest identity (97%) with the 16S rRNA gene of a *myroides sp*. strain (NCBI database, GQ: 383900). Based on these observations, the strains were identified as *Myroides sp.* The isolate had a 48% removal capacity of linuron at the 84^{th} day of incubation. The concerted action of *Myroides sp* has already been observed for degradation of atrazine by Smith *et al.,* 2006 reported that *Myroides sp* had the capacity to degrade atrazine herbicide with a removal percentage of 90% within 3 days. A study by Devers *et al.*, 2007 revealed that genes coding for atrazine degrading proteins are highly conserved, widespread and frequently associated with insertion sequences (IS) located on plasmids consequently, and the combination of IS-mediated rearrangement and plasmid transfer had been suggested to contribute to the assembly and dissemination of the atrazine-degrading capabilities in the environment.

From these earlier findings it may be possible for these genes to have contributed to strain LLa-1A capability to degrade the study compound linuron.

Phylogenetic analysis of 16S rRNA gene sequence of isolate LJk-5B showed that the isolate clustered with members of the genus *Xanthomonas sp*. phylogenetic analysis positioned isolate LJK-5B closely to *Xanthomonas bromi* with a sequence similarity of 98% and bootstrap values of between 50-100% certainly identifying isolate LJk-5B with *Xanthomonas sp*. The isolate had a 32% removal of linuron at the 84th day of incubation. Earlier studies by Oregaard and Sorensen 2007 reported that *Xanthomonas sp* had *merA* genes sequences which increased the functional diversity and the adaptation ability of the microbe to the environment bioremediation of Fluoranthene, a four-ring PAH, one of the principal PAHs in the environment.

The physiological and biochemical tests of LWa-2A, 16S rRNA gene sequencing and blast analysis indicated that LWa-2C was 99% homologous to 16S rRNA gene of *Arthrobacter* sp. A phylogenetic tree involving isolate LWa-2A revealed that LWa-2C was phylogenetically closest to *A. globiformis*. The isolate degraded 98.94 % of the initial concentration of 50mg/l of linuron over a period of 84 days when a plateau phase of growth was reached only very low and slow additional degradation occurred. In other related studies *Arthrobacter sp* have been reported to metabolize methoxy-methyl-substituted phenylureas such as linuron, monolinuron and metobromuron but not diuron and monuron (Widehem *et al.,* 2001). *Arthrobacter sp* has been has also been shown to degrade toxin swainsonine a compound found in locoweed (Medeiros *et al.,* 2003).

Isolate LWa-2C after Phylogenetic analysis of its 16S rRNA was seen to cluster closely with *Burkholderia cenocepacia* with a sequence similarity of 99%. Supported by most of the phenotypic and biochemical characteristics the isolates were identified with *Burkholderia sp*. The isolate had 95.54 % removal percentage of linuron by end of 84 day incubation period. *Burkholderia sp* have been reported to be among the most metabolically versatile microorganisms known, growing on more than 200 organic compounds. *Burkoholderia sp* are potentially reported to be important resource for bioremediation of chlorobenzene-contaminated sites (Beil, *et al.,* 1997). In bioaugmentation for remediation technology (Hong *et al.,* 2007) also reported that the bacterium had potential for use in bioremediation of fenitrothion and its metabolite-contaminated sites.

The GC-MS analysis of diazinon metabolites revealed that diazinon was broken down by different bacterial strains through desulfuration to yield metabolites like isopropyl methyl hydroxyl pyrimidine (diaxozon). The metabolite was detected in all samples tested in only the first 5 days of culture an indication that the metabolite was probably further decomposed to 2-Isopropyl – 4 – methyl -6 – hydroxyprimidine (IMHP) a less toxic metabolite. The observations are in line with those of Ohshiro *et al*., 1996 who isolated an *Arthrobacter* strain from a turf green soil that was able to rapidly hydrolyse diazinon among other OPs with the presumptive production of DETP and IMHP. On the 35^{th} day of culture among samples the tested a monoethylthiophosphate residue was detected. The presence of this metabolite could be as a result of the former metabolite DETP and IMHP further decomposition by phosphomonoesterases and

phosphodiesterases to release inorganic phosphate. The inorganic phoshate was assimilated by the bacterium as reported by Rosenberg and Alexander, (1979) in a diazinon-enrichment culture from sewage and soil samples which resulted in the isolation of a *Peudomonas putida* strain, which was able to use diazinon and other OP compounds like malathion, parathion and dimethoate as phosphorus sources.

The general metabolic pathway for metabolism of aniline involves oxidative deamination to give catechol, which may be further degraded by different ring cleavage pathways Lyons *et al.,* (1984) however, various substitutions to the ring structure, for example halogen or nitro groups, may prevent or delay complete mineralization. GC-MS spectrums from linuron degradation among isolates tested in this study revealed that all the isolates had the ability to degrade the compound to five main metabolites namely: N-O dimethylhydroxylamine, 3-chloroanalline, 4-chloroanlline, 3,4 dichloroanalline and N-methyl-N-methxy-carbamic acid however, no single strain had the ability to degrade these metabolites further over a culture period of 84 days. This is congruent with El-Fantroussi, (2000) in a linuron aniline metabolites cross feeding experiment where attempts to isolate pure cultures able to degrade the aniline derivatives were unsuccessful.

5.2 Conclusion and Recommendations

5.2.1 Conclusion

The study has demonstrated that farms under horticultural production survey applied different pesticides formulations in their farms. In this study 18 diazinon and 13 linuron degrading isolates were obtained from soils of horticultural farms in four sampled regions of Kenya. The HPLC and GC-MS degradation results for diazinon and linuron in liquid medium indicated that microbial degradation is one of mechanisms of diazinon and linuron dissipation in *invitro*. Phylogenetic analysis allowed clustering diazinon and linuron degrading isolates into six and eight different genera respectively.

5.2.2 Recommendations

5.2.2.1 Recommendations from the findings of the study

From the results of the study I would recommend as follows;

1. Isolates; DJk-4A from the genera *Pseudomonas,* DKi-6A from *Staphylococcus,* DLoG-8A from *paracoccus,* DSh-3A from *Klebsiella,* DKa-5C from *enterobacter* and DSh-3C from *Proteus* could be used *invivo* in bioremediation of soils under horticultural production contaminated with diazinon since they showed high degradation potential.

2. Isolates; LLa-1A from the genera *Myriodes* LoG-8A from *Lysinibacillus,* LWa-2A from *Arthrobacter,* LWa-2C from *Burkholderia,* LKi-3A from *Stenotrophomonas* LJk-5C from *Pseudomonas* and LJk-5B from *Xanthomonas* could be used in bioremediation of soils under horticultural production contaminated with linuron since they showed over 70% biodegradation potential.

5.2.2.2 Recommendations for further studies

1. To get a clear insight into the mechanism of biodegradation, further studies should be done to characterize and identify the proteins with biodegradative activity. That information would be important in molecular cloning of the genes coding for such proteins.
2. The most highly active isolates should be further studied and subjected to varying concentration of pesticides in an attempt to determine possible threshold concentrations in these systems for their growth on contaminants and bioremediation.
3. Research on synergistic consortia involved in complete linuron mineralization should be undertaken to clearly understand the interactions between consortia.

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APPENDICES

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

QUESTIONNAIRE FOR AGROCHEMICAL SURVEY

Questionnaire No. _____ **Date** _____

1. Name of farm _____

2. District_____**Division**_____

3. Contact person _____**Title** _____

4. Telephone _____ **Email** _____

5. Address PO BOX _____ **FAX** _____

6. Crops grown a) Fruits b) Vegetables c) Flowers

7. Specific crops _____, _____, _____

8. Agrochemicals used

Target pests for each		**Pesticide (Trade name)**
i)	_____	_____
ii)	_____	_____
iii)	_____	_____
iv)	_____	_____
v)	_____	_____

9. Class of chemicals

_____
_____
_____
_____
_____
_____

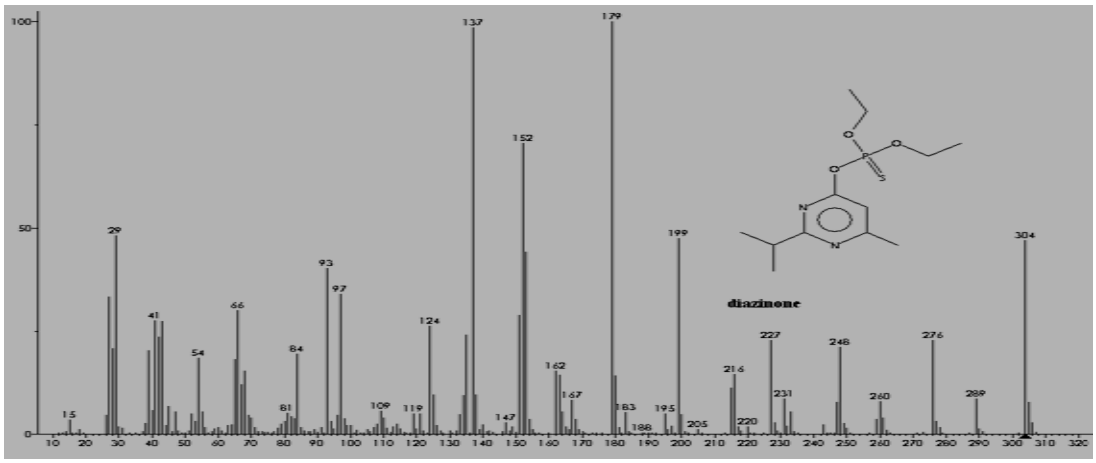
10. Methods of waste water disposal (if any)

a) **Wet lands**
b) **Drain into ditches**
c) **None**
d) **Other**

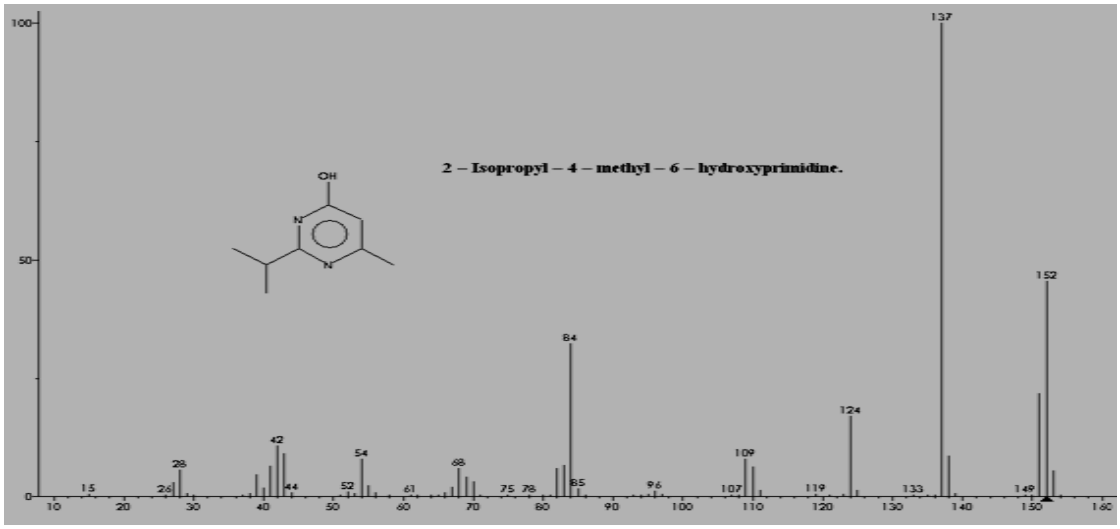
11. Any visible Agrochemical effect on the environment

_____

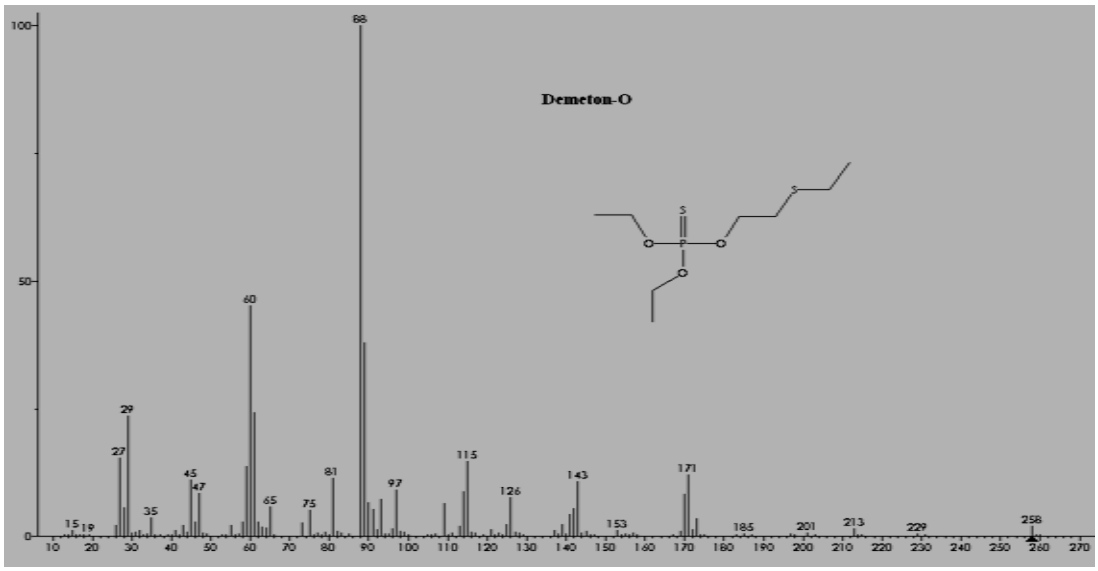
Appendix 1: Survey instrument for pesticides applications in horticulture farms



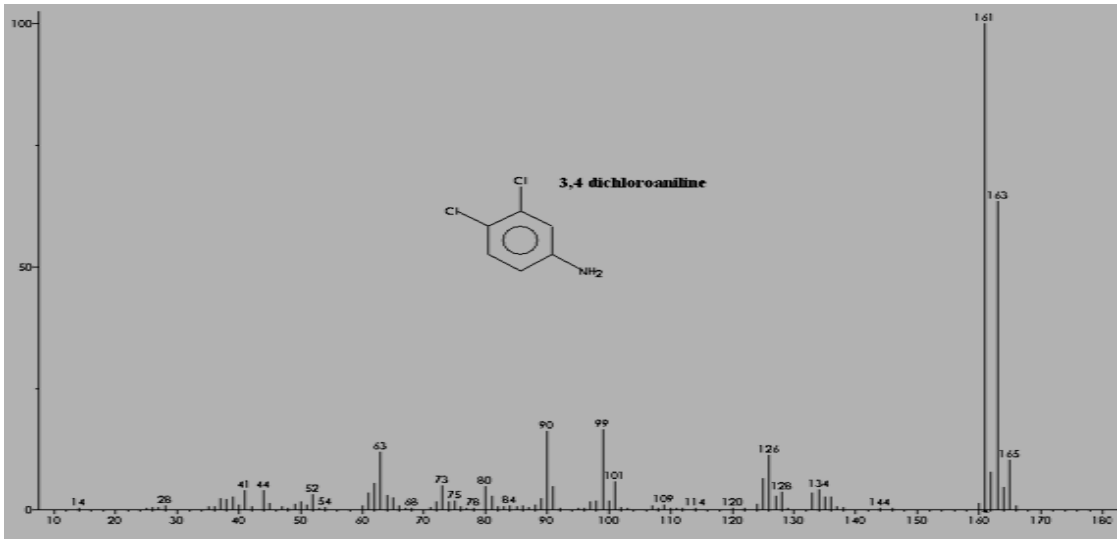
Appendix 2a: GC-MS spectrum of Diazinone



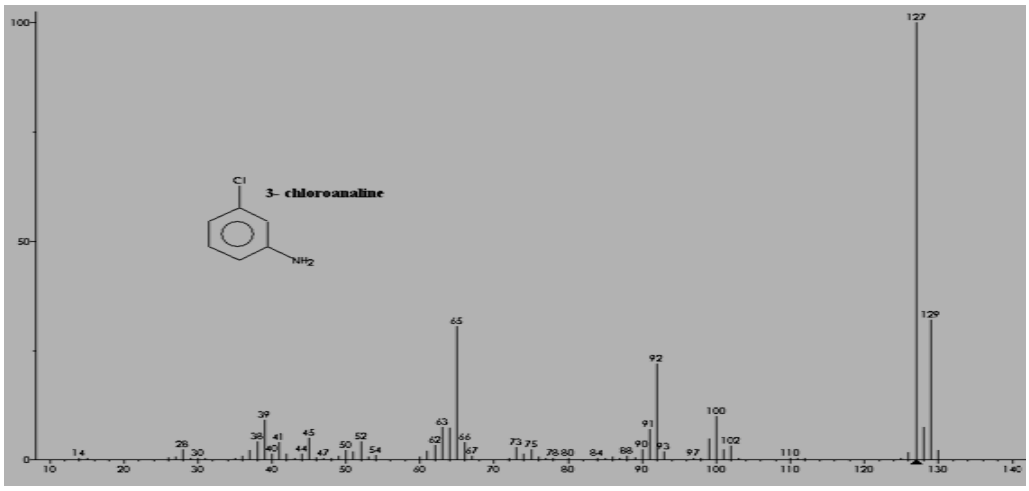
Appendix 2 b: GC-MS spectrum of 2 - Isopropyl -4- methyl – 6 - hydroxyprimidine



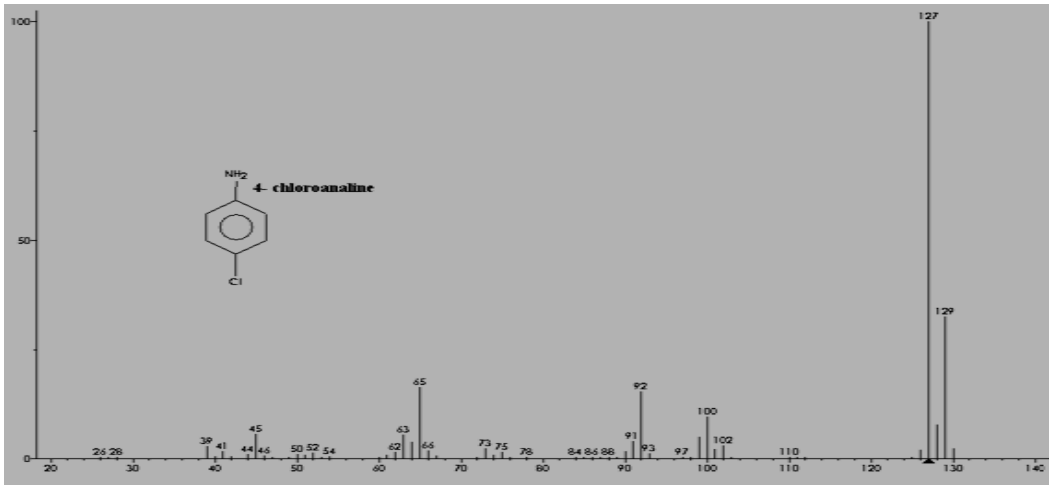
Appendix 2c: GC-MS spectrum of O,O-Diethyl O-[2-(ethylthio)ethyl] phosphorothionate (Demeton-O)



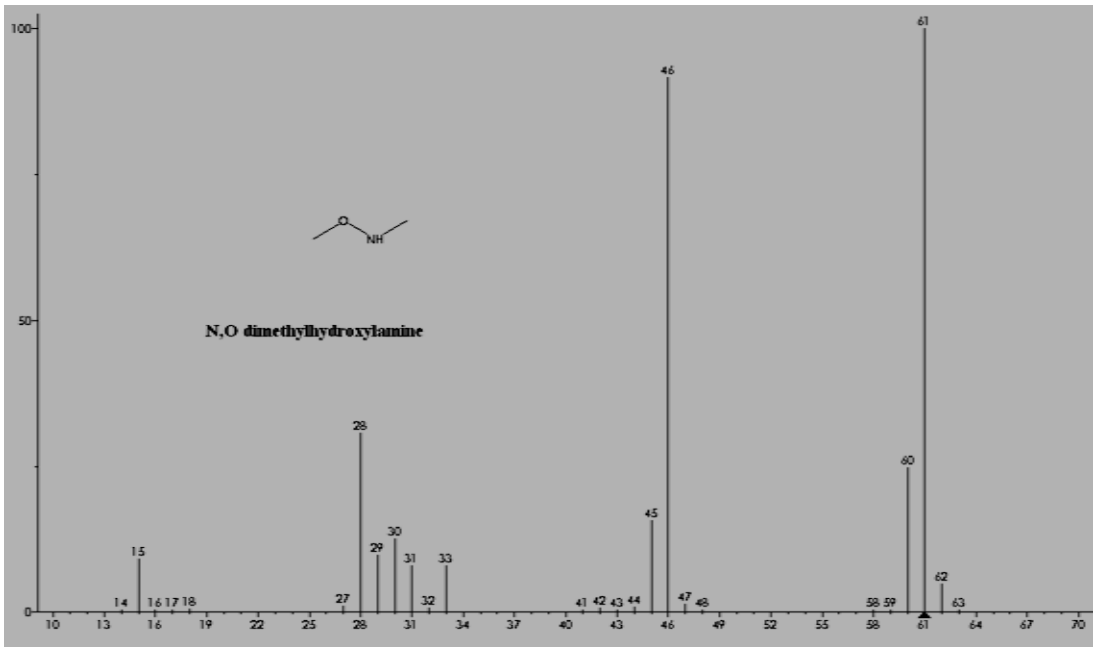
Appendix 2 d: GC-MS spectrum of 3,4 dichloroanilline



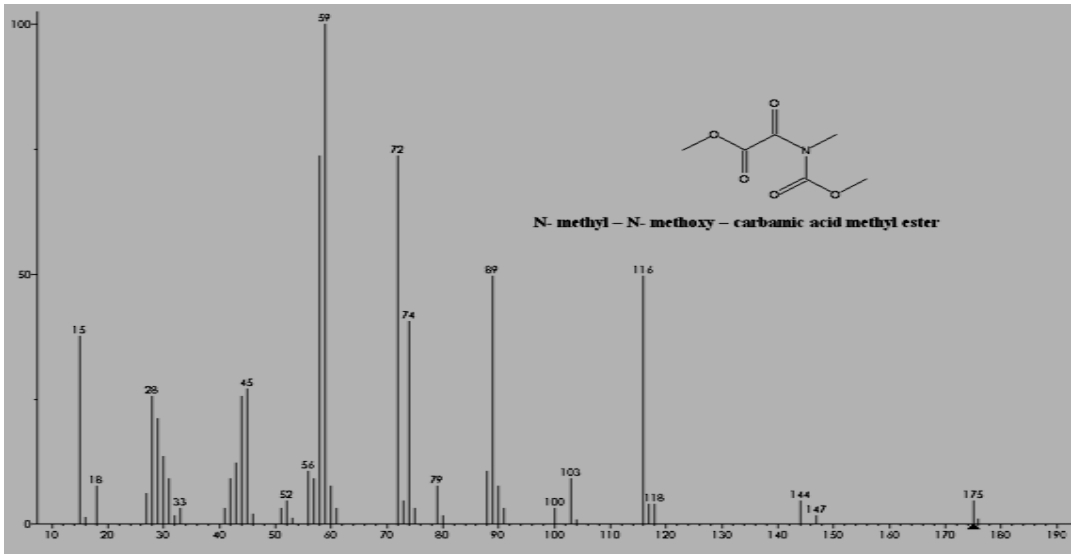
Appendix 2 e: GC-MS spectrum of 3- chloroanilline



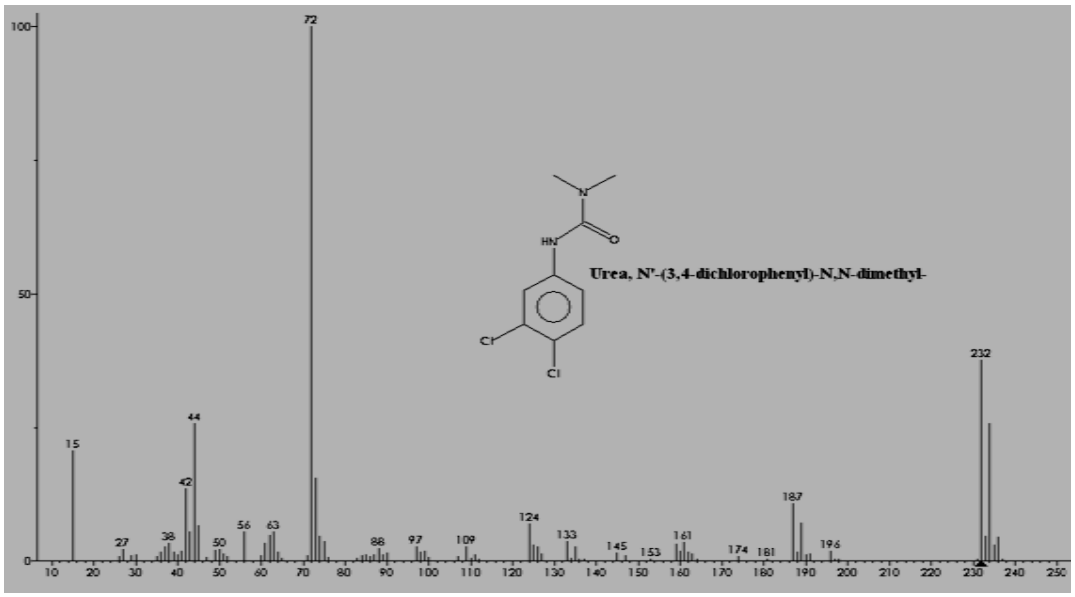
Appendix 2 f: GC-MS spectrum of 4- chloroanilline



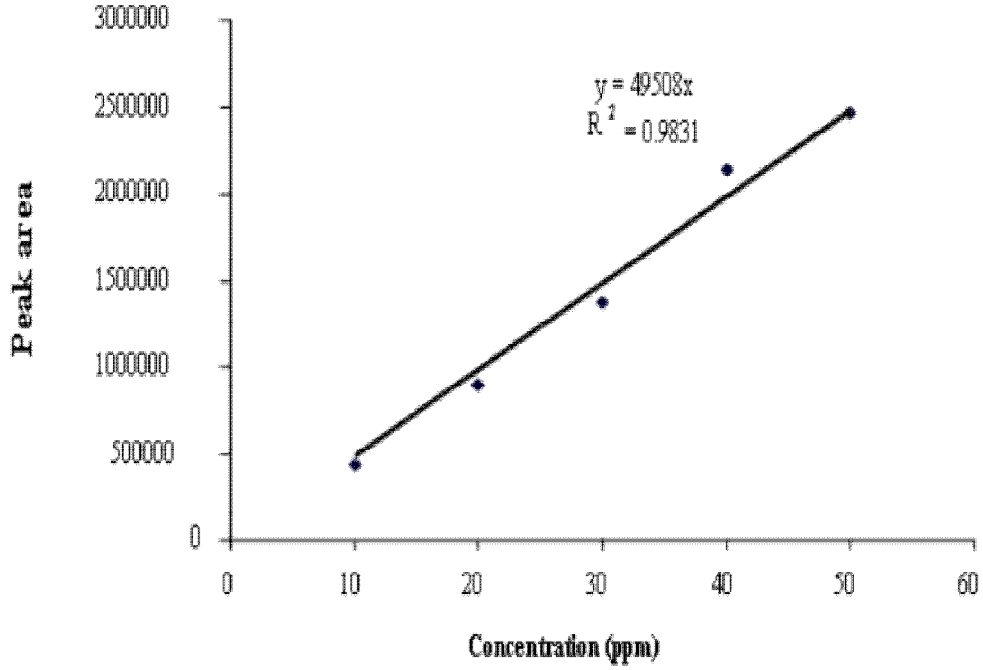
Appendix 2 g: GC-MS spectrum of N,O dimethylhydroxylamine



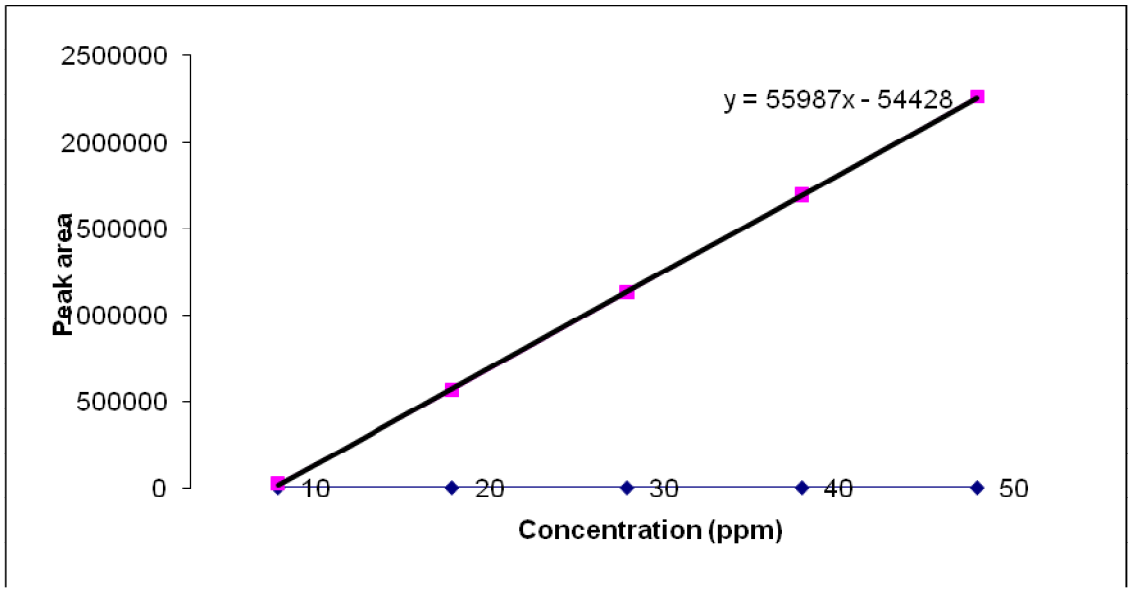
Appendix 2h: GC-MS spectrum of N-methyl-N-methoxy-carbamic acid methyl ester



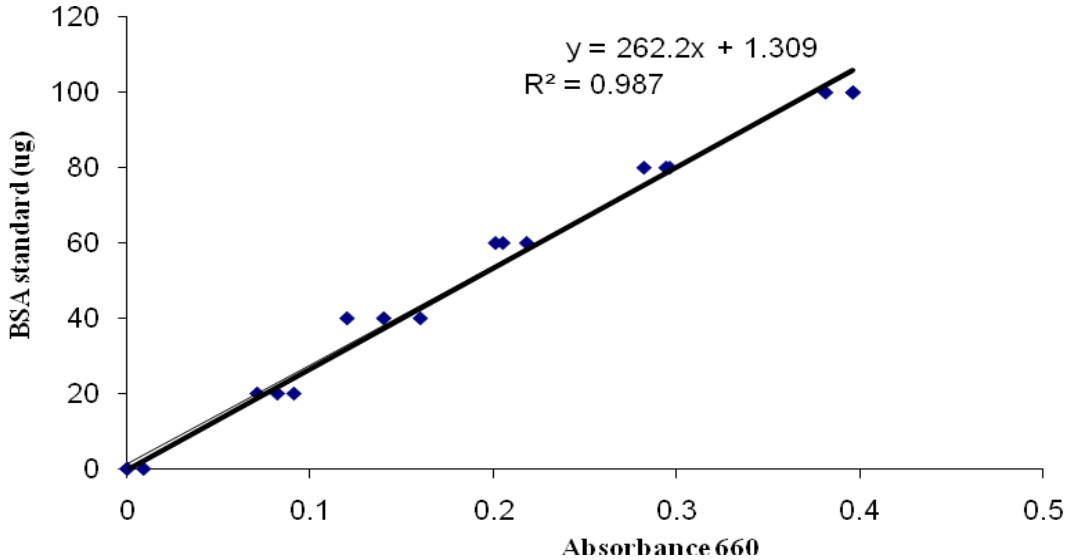
Appendix 2 i: GC-MS spectrum of urea, N (3,4-dichlorophenyl)-N-N dimethyl



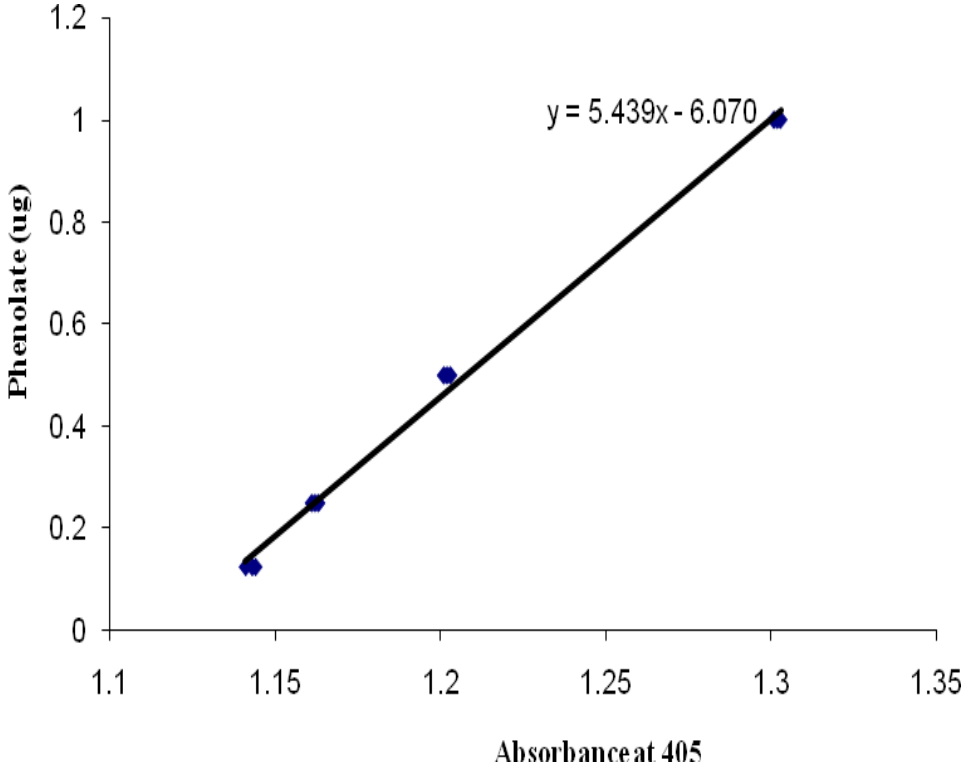
Appendix 3a: Standard curve for linuron



Appendix 3 b: Standard curve for diazinon



Appendix 3 c: Lowry total protein assay standard curve



Appendix 3 d: p-Nitrophenolate assay standard curve

Appendix 4: Mean degradation level of diazinon

	Concentration of diazinon (ppm)							
Isolates	At0 day	At 5 days	At 10 days	At 15 days	At 20 days	At 25 days	At 30 days	At 35 days
Control	$12\pm0.0^{a}{}_{a}$	$11.53\pm0.025^{a}{}_{a}$	$10..900\pm0.034^{a}{}_{b}$	$10.38\pm0.055^{a}{}_{b}$	$9.92\pm0.065^{a}{}_{c}$	$9.52\pm0.001^{a}{}_{c}$	$9.28\pm0.041^{a}{}_{c}$	$9.13\pm0.056^{a}{}_{c}$
DSh-3A	$12\pm00^{a}{}_{a}$	$11.53\pm0.087^{a}{}_{b}$	$9.90\pm0.092^{b}{}_{c}$	$6.38\pm0.066^{e}{}_{d}$	$4.92\pm0.0195^{e}{}_{e}$	$3.52\pm0.009^{d}{}_{f}$	$2.58\pm0.005^{d}{}_{g}$	$2.53\pm0.001^{d}{}_{g}$
DSH-3C	$12\pm0.0^{a}{}_{a}$	$10.67\pm0.543^{b}{}_{b}$	$8.67\pm0.065^{bc}{}_{c}$	$7.06\pm0.045^{d}{}_{d}$	$5.54\pm0.065^{d}{}_{e}$	$5.15\pm0.012^{b}{}_{e}$	$4.05\pm0.034^{b}{}_{f}$	$4.06\pm0.023^{b}{}_{f}$
DJk-4A	$12\pm0.0^{a}{}_{a}$	$11.54\pm0.032^{a}{}_{b}$	$7.97\pm0.056^{d}{}_{c}$	$5.54\pm0.000^{f}{}_{d}$	$3.65\pm0.034^{f}{}_{f}$	$2.43\pm0.006^{e}{}_{f}$	$1.76\pm0.110^{f}{}_{g}$	$0.57\pm0.004^{f}{}_{h}$
LoG-8A	$12\pm0.0^{a}{}_{a}$	$11.61\pm0.043^{a}{}_{b}$	$9.65\pm0.032^{b}{}_{c}$	$8.87\pm0.021^{b}{}_{d}$	$7.98\pm0.033^{b}{}_{e}$	$5.32\pm0.032^{b}{}_{f}$	$3.54\pm0.003^{c}{}_{g}$	$2.87\pm0.032^{c}{}_{h}$
Dki-6A	$12\pm0.0^{a}{}_{a}$	$9.03\pm0.005^{a}{}_{b}$	$8.07\pm0.076^{cd}{}_{c}$	$7.98\pm0.054^{c}{}_{d}$	$6.98\pm0.003^{c}{}_{e}$	$4.76\pm0.016^{c}{}_{f}$	$2.21\pm0.003^{e}{}_{g}$	$0.76\pm0.001^{e}{}_{h}$
DJk-4C	$12\pm0.0^{a}{}_{a}$	$8.32\pm0.004^{d}{}_{b}$	$6.83\pm0.005^{e}{}_{c}$	$5.01\pm0.004^{g}{}_{d}$	$4.54\pm0.005^{e}{}_{e}$	$3.76\pm0.087^{d}{}_{f}$	$3.67\pm0.043^{c}{}_{f}$	$2.85\pm0.004^{c}{}_{g}$
CV%	2.4%	2.44%	2.3%	2.4%	2.4%	2.4%	2.5%	1.8%
$LSD_{0.05}$	-	0.049	0.463	0.252	0.369	0.343	0.247	0.099

Mean values within columns (a) and rows (a) followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$) $^{a,b,\ c,\ d\ and\ e}$ Values of days and concentration corresponds to means (±SE) of three independent experiments.

Appendix 5: Mean degradation level of linuron

Concentration of linuron (ppm)							
Isolates	At 0 day	At 7 days	At 14 days	At 21days	At 28 days	At 35 days	At 42 days
Control	$50\pm0.004^{a}{}_{a}$	$49.85\pm0.001^{a}{}_{a}$	$49.75\pm0.005^{a}{}_{a}$	$48.25\pm0.009^{a}{}_{b}$	$47.73\pm0.014^{a}{}_{b}$	$47.85\pm0.037^{a}{}_{b}$	$45.41\pm0.003^{a}{}_{c}$
Lla-1A	$50\pm0.001^{a}{}_{a}$	$48.54\pm0.002^{b}{}_{b}$	$44.45\pm0.095^{c}{}_{c}$	$42.98\pm0.015^{b}{}_{d}$	$33.36\pm0.064^{d}{}_{e}$	$29.27\pm0.055^{e}{}_{f}$	$26.54\pm0.009^{d}{}_{g}$
LJk-5C	$50\pm0.00^{a}{}_{a}$	$43.69\pm0.003^{d}{}_{a}$	$37.38\pm0.005^{e}{}_{c}$	$31.16\pm0.071^{f}{}_{d}$	$27.11\pm0.054^{f}{}_{e}$	$22.59\pm0.081^{g}{}_{f}$	$15.41\pm0.043^{f}{}_{g}$
Lwa-2C	$50\pm0.003^{a}{}_{a}$	$47.43\pm0.004^{a}{}_{b}$	$40.27\pm0.005^{d}{}_{c}$	$40.54\pm0.001^{d}{}_{d}$	$29.32\pm0.064^{e}{}_{e}$	$25.68\pm0.077^{f}{}_{f}$	$18.79\pm0.024^{e}{}_{g}$
LOG-8A	$50\pm0.00^{a}{}_{a}$	$48.54\pm0.005^{b}{}_{b}$	$45.33\pm0.006^{b}{}_{c}$	$42.78\pm0.009^{b}{}_{d}$	$40.43\pm.0032^{b}{}_{e}$	$37.21\pm0.034^{b}{}_{f}$	$33.71\pm0.002^{b}{}_{g}$
LJk-5B	$50\pm0.007^{a}{}_{a}$	$43.69\pm0.022^{e}{}_{b}$	$40.36\pm0.076^{d}{}_{c}$	$37.64\pm0.055^{e}{}_{d}$	$35.46\pm0.087^{c}{}_{e}$	$33.33\pm0.051^{c}{}_{f}$	$32.09\pm0.055^{c}{}_{g}$
Lwa-2A	$50\pm0.002^{a}{}_{a}$	$47.43\pm0.003^{c}{}_{b}$	$45.21\pm0.021^{b}{}_{c}$	$40.54\pm0.031^{c}{}_{d}$	$35.32\pm0.065^{c}{}_{e}$	$30.82\pm0.061^{d}{}_{f}$	$26.04\pm0.001^{d}{}_{g}$
CV %	2.36	2.36	2.36	2.36	2.36	2.36	2.36
$LSD_{0.05}$	0.00	0.344	0.72	0.38	0.683	0.509	0.895
	At 49 days		At 56 days	At 63 days	At 70 days	At 77 days	At 84 days
Control	$44.35\pm0.015^{a}{}_{d}$		$42.45\pm0.002^{a}{}_{e}$	$41.62\pm0.007^{a}{}_{f}$	$41.35\pm0.064^{a}{}_{ef}$	$40.55\pm0.045^{a}{}_{g}$	$40.75\pm0.078^{a}{}_{g}$
LLa-1A	$22.75\pm0.087^{d}{}_{h}$		$17.61\pm0.065^{d}{}_{i}$	$12.51\pm0.065^{d}{}_{j}$	$7.69\pm0.051^{d}{}_{k}$	$4.59\pm0.054^{c}{}_{l}$	$3.11\pm0.088^{d}{}_{m}$
LJk-5C	$9.04\pm0.061^{f}{}_{h}$		$4.91\pm0.067^{g}{}_{i}$	$0.89\pm0.053^{g}{}_{jk}$	$0.58\pm0.065^{g}{}_{kl}$	$0.41\pm0.051^{e}{}_{j}$	$0.32\pm0.033^{f}{}_{l}$
Lwa-2C	$14.74\pm0.041^{e}{}_{h}$		$9.54\pm0.065^{f}{}_{i}$	$5.84\pm0.004^{f}{}_{j}$	$3.56\pm0.008^{f}{}_{k}$	$2.25\pm0.011^{d}{}_{l}$	$2.23\pm0.054^{e}{}_{l}$
LOG-8A	$31.21\pm0.099^{b}{}_{h}$		$27.43\pm0.074^{b}{}_{j}$	$23.73\pm0.068^{b}{}_{j}$	$18.65\pm0.067^{b}{}_{k}$	$14.65\pm0.078^{b}{}_{l}$	$9.43\pm0.003^{c}{}_{m}$
LJk-5B	$29.26\pm0.072^{c}{}_{h}$		$24.53\pm0.054^{c}{}_{i}$	$20.45\pm0.041^{c}{}_{j}$	$16.33\pm0.071^{c}{}_{k}$	$14.87\pm0.005^{b}{}_{l}$	$14.15\pm0.044^{b}{}_{m}$
Lwa-2A	$22.57\pm0.006^{d}{}_{h}$		$14.43\pm0.001^{e}{}_{i}$	$9.26\pm0.051^{e}{}_{j}$	$5.94\pm0.032^{e}{}_{k}$	$2.65\pm0.006^{d}{}_{l}$	$0.53\pm0.091^{f}{}_{m}$
CV %	2.36		2.36	2.36	2.36	2.36	2.36
$LSD_{0.05}$	0.913		0.519	0.714	0.973	0.491	0.53

Mean vaues within a columns and rows followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$) [a,b and c] Values of days and concentration corresponds to means (±SE) of three independent experiments