ASSESSMENT AND MOLECULAR CHARACTERIZATION OF BACTERIAL DEGRADERS OF HEXAZINONE AND 2,4-D HERBICIDES FROM SUGARCANE CULTIVATED SOILS IN NZOIA COMPANY NUCLEUS ESTATES, KENYA

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A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of the Award of Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY
OCTOBER, 2018

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted for examination in any institution

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RECOMMENDATION

This thesis has been submitted for examination with our approval as university supervisors

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DEDICATION

This work is dedicated to my mother Justa Njue for steering me in the ways of Christ Jesus without which I could have not made it this far.

ACKNOWLEDGEMENT

Glory and honor to almighty God for granting me good state of health and abundant grace to pursue my MSc study.

I would like to express my profound gratitude to my supervisors Dr. A. Ngigi for her selfless and tireless guidance in chemistry work, Dr. A Muia for guidance in microbiology work and Dr. Silas Kiruki for his guidance in biochemistry and molecular work. Special thanks to the National Council for Science Technology and Innovation (NACOSTI) for funding this project. I also thank the Head of the Department of Biological Sciences Egerton University, for allowing me to use their laboratory facilities for dehydrogenase assay and microbial isolation.

I am grateful to Ms. C. Kariuki and Mr. L. Mungai of Department of Biological Sciences, Egerton University, for their assistance in microbiology and dehydrogenase analysis. I also thank Mr. Rotich of Jomo Kenyatta University of Agriculture and Technology (JKUAT) for technical assistance in molecular work and Ms. A. Muyale of Masinde Muliro University of Science and Technology (MMUST) for her assistance in HPLC analysis.

I would also like to extend my appreciation to the Department of Animal Science at Egerton University and in particular Mr. M. Mutumba, for allowing me to use their Soxhlet apparatus and Department of Chemistry for giving me an opportunity to use their rotary evaporator. I am also grateful to the Department of Soil Science for allowing the use their facilities for the analysis of soil physicochemical parameters.

My appreciations to my brother Laban Njeru and his family for selfless compassion, motivation and inspiration throughout my studies. Special thanks to all my siblings who stood with me in prayer and encouragement.

My appreciation also goes to my student colleagues T. I. Kavuli, S. Ngure, P. Kirianki, J. Rono, E. Ogola and V. Wekesa with whom we shared ideas and knowledge during period of this study. Last but not least, my special appreciations to my wife Mary Wambui for her support through prayer, moral support and encouragement during the entire work.

ABSTRACT

Agrochemicals have been used extensively all over the world for improved food security, industrial development, and poverty reduction. In Western Kenya, herbicides are used to clear weeds in sugarcane growing plantations. Uncontrolled and unregulated use of these herbicides results in contamination of both soils and the associated drainage systems. Their use may have adverse effects such as disruption of microbial, animal and plant diversity in addition to serious effects to human health. Repeated application of the herbicides results in biochemical adaptation of native microbes especially the bacteria, which in turn lead to the enhanced mineralization of the herbicides. Hexazinone and 2,4-dichlorophenoxyacetic acid (2,4-D), are among the most commonly used herbicides in Nzoia sugar cane farms in Western Kenya. The main objective of this study was to isolate and characterize bacterial degraders of hexazinone and 2,4-D from soils collected from Nzoia Sugar Company sugarcane farms in Western Kenya and also to determine their effects on colony forming units (CFUs) and total dehydrogenase (DHA) activity. Isolation was achieved through incubation experiments in mineral salt medium amended with the herbicides. Growth of isolates indicated by turbidity of broths was monitored by optical density measurements, whereas degradation by isolates was determined by quantification of residual herbicides using high performance liquid chromatography. DHA activity analysis was achieved through triphenyl tetrazolium chloride (TTC) based method. The DHA activity showed that 2,4-D had inhibitory effects on total DHA activity while hexazinone had boosting ability for the bacterial growth. Degradation experiments yielded four bacteria strains encoded as N13010H1, N15030H2, N15030H3 and N212H4 which were able to degrade hexazinone and three bacteria degraders encoded as N139D1, N13010D3 and N13010D4 with potential to degrade 2,4-D. Biochemical and molecular characterization showed that, N13010H1 was *Bacterium* NLAE zl-H322. N15030H2 was identified as *Enterobacter* sp, N15030H3 as *Bacillus cereus* while N212H4 was identified as *Staphylococcus aureus.* All the four isolates had high potential for hexazinone degradation ranging from 57.6 to 82% with N15030H3 showing the highest potential. For 2,4-D degraders, N13010D3 was identified as *Serratia marcescens,* while N139D1 and N13010D4 were identified as *Bacillus* sp and Uncultured bacterium clone, respectively. The biodegradation capacity of 2,4-D by the three isolates ranged from 65 to 82% with N13010D3 showing the highest potential. The isolates can be modified and utilized for bioremediation of hexazinone and 2,4-D contaminated soils.

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LIST OF ABBREVIATIONS AND ACRONYMS

2,4-D	2, 4-Dichlorophenoxyacetic Acid
3,5-DCC	3,5-Dichlorocatechol
ATP	Adenosine Triphosphate
BEE	Butoxy Ethyl Ester
BLAST	Basic Local Alignment Search Tool
CAS	Chemical Abstracts Service
DEA	Diethyl Amine
DMAS	Dimethylamine Salt
DNA	Deoxyribonucleic Acid
dNTPs	deoxynucleotide triphosphate
EHE	Ethylhexyl Ester
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IPA	Isopropyl Acid
IPE	Isopropyl Ester
K_{OC}	Organic Carbon Adsorption Coefficient
K_{OW}	Octanol/water coefficient
MBC	Mixed Bacteria Culture
MSM	Mineral Salt Medium
NCBI	National Council for Biotechnology Institute
NHL	Non-Hodgkin’s Lymphoma
OD	Optical Density
OM	Organic Matter
PCR	Polymerase Chain Reaction
PTFE	Polytetraflouroethylene
rRNA	ribosomal Ribonucleic Acid
TAE-buffer	Tris-Acetate Buffer
TIPA	Triisopropyl Acid
TPF	TriphenyltetrazoliumFormazan
TTC	Triphenytetrazolium Chloride
USDA-ARS	US-Department of Agriculture-Agriculture Research Service
UV-VIS	Ultra Violet-Visible Spectra

WHO	World Health Organization

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CHAPTER ONE
INTRODUCTION

1.1 Background information

Agricultural production is one of the largest and most important economic activities in the world, and particularly in the third world countries, where agriculture has a significant impact on gross domestic product (GDP) growth. The use of agrochemicals is a major contributing factor in agricultural production (Aseno, 2008) for increased produce. However, the extensive use of agrochemicals in agriculture compromises soil and water quality thus raising a number of environmental concerns. One major concern is the contamination of soil and water resources (Younes and Galal-Gorchev, 2000). Agrochemicals are some of the major causes of water pollution, whereas some agrochemicals are persistent organic pollutants and contribute to soil contamination.

Sugarcane (*Saccharum officinarum*), which is a grass plant belonging to the family *Graminae* and grown in tropical and sub-tropical parts of the world has gained high global demand due to its wide use, such as energy, bio-ethanol and sugar production (Rabelo *et al*., 2011). Sugarcane is a semi-perennial crop with high labor demands especially on weeding, making it a high user of agrochemicals alternatives such as herbicides (Vieira *et al*., 2012). Some of the known herbicides used to control weeds in sugar cane plantations include roundup (glyphosate), metribuzin, hexazinone and 2,4-D (Srivastava and Chauhan, 2006). In Kenya hexazinone and 2,4-D are among the most frequently used herbicides for weed control in sugarcane farming in nuclear estates of Nzoia Sugar Company.

Considering the widespread use of pesticides in soil, their fate is of great concern, as they pose a major threat to human health, quality of soil, air, surface and ground water resources. Degradation is among the key processes that affect the fate and transport of pesticides in the environment. It is considered as a fundamental attenuation process for pesticides in soil. This process is catalyzed by soil microbes and is affected by a variety of interactions among microorganisms, various soil constituents and the specific pesticide involved (Boivin *et al*., 2005) as well as physical-chemical conditions of soil such as radiation, water, temperature, weathering among others (Monteiro and Boxall, 2009).

Hexazinone (3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4-dione) is a broad-spectrum triazine herbicide used for the control of weeds in various crops such as sugarcane, tea, coffee and horticultural nurseries (Vandervoort *et al*., 1997; Wang *et al*., 2005). This herbicide has a low K_{oc}, which is an indication of low adsorption into the organic matter in the soil and therefore making it highly soluble in water and of great potential for

leaching and partitioning into water more than to soil (Ganapathy 1996; Wang *et al*., 2006). Hexazinone has moderate to long half-life of about 90 days (Zhang *et al*., 2002). 2,4-dichlorophexoyacetic acid, on the other hand, is a polychlorinated aromatic hydrocarbon herbicide that has been widely used throughout the world since 1940s to control broadleaf weeds and woody plants (IARC, 1997). This herbicide exhibits same close physicochemical characteristics to those of hexazinone such as high soil mobility due to low K_{oc} (Gervais *et al*., 2008).

Several studies have been carried out on effects of the two herbicides on human health and aquatic lives as well. These studies have elucidated some of serious effects of 2,4-D which are carcinogenicity, teratogenicity, immunosuppression, neurotoxicity, hepatotoxicity to human and other animals. On the other hand, hexazinone has been found to have negative effects on zooplanktons and phytoplankton community (Tuschl and Schwab, 2003). Despite much having been documented on the effects of these herbicides on non-targeted organisms and human beings, equal attention needs to be paid on their fate in soils.

The persistent use of herbicides in the environment may lead to microorganisms developing an adaptation mechanism. This is enhanced by diverse microbial community and their tremendous fine-tuning rate of mutation, which optimizes their survival to the constantly changing environmental condition (Denamur and Matic, 2006). The proliferation of microorganisms that use pesticides as carbon or nitrogen source, or both, for the growth leads to mineralization of these pesticides (Racke and Coats, 1990). At present, biological decontamination of pesticide residues has become an increasingly important area of research and it is preferable if microbial or biological methods of degradation are available for the various pesticidal compounds (Wang *et al*., 2005).

In Kenya, studies have shown the presence of pesticide residues in sugarcane cultivated soils and water from the drainage basins in sugarcane farming regions, hence the growing concerns regarding the potential effects of pesticides on non-targeted organisms (Getenga *et al*., 2004). It is important to carry out routine environmental monitoring of pesticides residue in order to determine safe levels of those pesticides that may or do cause health problems. Considering there is a number of environmental fate of pesticides residues such as hydrolysis, photo-degradation, transfer and biodegradation environmental monitoring should be accompanied by establishment of the fates of these pesticides if their residues are detected (Kashyap *et al*., 2005). However, studies have shown that biodegradation is the most effective and ecofriendly fate of pesticides owing to the fact that there are minimal toxic

products released (Wang *et al*. 2006). This has necessitated the need to carry out studies on bacteria that can mineralize these pesticides in soils of Nzoia River drainage basin.

1.2 Statement of the problem

Continued use of pesticides in an effort to increase agricultural production has resulted in contamination of the environment. Hexazinone and 2,4-D, which are among the most frequently used herbicides in sugarcane plantations, have been reported to have adverse effects on human health, primary producers, soil microorganisms such as nitrogen fixers, and on non-targeted plants. Several studies have shown depression of phytoplankton such as algae, which play a very vital role at the base of aquatic food chain by hexazinone. Consequently, several researches have attributed this to reduction of zooplankton community such as fish. 2,4-dichlorophenoxyacetic acid has been linked to carcinogenic, teratogenic and immune suppression effects in human and other animals on areas where it is commonly applied. In Western Kenya where sugarcane production is common, the use of hexazinone and 2,4-D is rampant for reduction of labor costs and increased yields. Native bacteria normally occurring in low concentrations in soil have been shown to degrade pesticides and other chemicals. Such microbes have potential for use in bioremediation of contaminated environments. Presence of such pesticide adapted microorganisms has barely been investigated in the study area and in Kenya in general. This study therefore aims at isolating and characterizing bacteria capable of degrading hexazinone and 2,4-D using selective enrichment, biochemical and molecular techniques. Such bacteria can be useful in providing an ecofriendly method of decontamination of the environment. In addition, the effect of the two herbicides on soil microbial functions and activities was evaluated using soil CFUs and DHA assays.

1.3 Objectives

1.3.1 General objective

To characterize bacterial degraders of hexazinone and 2,4-D herbicides from sugarcane cultivated soils from Nzoia sugar company nucleus estate.

1.3.2 Specific objectives

1. To determine soil physicochemical characteristics and residual hexazinone and 2,4-D in soils of selected sugarcane farms from Nzoia sugar company nucleus estates.
2. To determine CFUsand enzyme dehydrogenase activity following application of herbicides in the farms.

3. To isolate bacteria capable of degrading hexazinone and 2,4-D from selected sugarcane farms of Nzoia sugar company nucleus estates.
4. To determine the rate of hexazinone and 2,4-D degradation using single and mixed bacteria isolates.
5. To carry out biochemical and molecular characterization of isolated degraders of hexazinone and 2,4-D.

1.4 Hypotheses

1. Soils from selected sugarcane farms from Nzoia sugar company nucleus estates have different physicochemical characteristics and contained detectable hexazinone and 2,4-D residues.
2. Application of the two herbicides affects the soil CFUs and dehydrogenase enzymatic activity.
3. Soils from Nzoia sugarcane farms contain different bacteria capable of degrading hexazinone and 2,4-D herbicides.
4. There is significance difference in rate of degradation of the two herbicides by single organism and mixed bacterial cultures.
5. The biochemical and molecular identities of the isolated degraders are different.

1.5 Justification

As the world industrialization rapidly grows and the world population continues increasing, there is coupled increase in use of synthetic chemicals such as agrochemicals to improve agricultural production to sustain the population growth. Despite the positive impacts in agriculture, their adverse effect to the environment is of great concern. These environmental effects of pesticides go far beyond the locality where they are used. This is due to surface run-off and leaching processes, which transfer pesticides residues into water bodies such as rivers and lakes. Some of these pesticides inflict serious harm and health problems to humans as well as to the biodiversity. It therefore calls for remarkable efforts to implement new technologies to reduce or eliminate these contaminants from the environment. One promising area that is currently being explored and that has not been well exploited is to investigate potential degradation of these pesticides by native microflora and the use of adapted microorganisms to remove pollutants from contaminated sites. The use of microorganism to decontaminate polluted site referred to as bioremediation is an effective, minimally hazardous, economical, versatile, and environment-friendly strategy. Microorganisms have the ability to transform and/or degrade pesticides, as an adaptation

mechanism to survive where these chemicals have been applied for a long time. In addition, in the presence of alternative carbon source these microbes are able to breakdown the chemical pollutant using non-specific enzyme resulting to the cleanup of such chemicals. Microorganisms' capability to develop an alternative metabolic pathway enhances this degradation. Since the utilization of these compounds is to derive energy, the microorganisms use them widely without production of toxic compounds when complete mineralization is achieved. The aim of this study was to isolate and identify bacteria that can degrade hexazinone and 2,4-D in selected soils from Nzoia sugar company nucleus estate. The nucleus estate drainstheir run-off into Kanywa river which is one of major river Nzoia tributary. River Nzoia traverses several counties before it drains into Lake Victoria which is of international importance. From the study the 16S rRNA gene sequencing of the isolated degraders of the two xenobiotics showed availability of naturally occurring microbes that can be used for bio-remedy in place where such herbicides are applied.

CHAPTER TWO
LITERATURE REVIEW

2.1 Sugarcane farming in Kenya

In Kenya, agriculture sector is the backbone of the country economy (World Bank Research, 2007). It is by far the single largest economic sector in Kenya and accounts for about 30% of GDP, over 60% of the exports, 75% of the total labour force and provides 80% of industrial raw materials (Economic Survey, 2007; Chesterman and Neely, 2015).

In the agriculture sector, sugarcane is an important agro-industrial crop in the tropics and sub-tropics due to its high sucrose content and bioenergy potential (FAO, 2010; Suman, Kirtiraj, and More,2014). Sugarcane growing is one of the subsectors that contributes to the national economy (Guda *et al*., 2001; Chesterman and Neely, 2015) and supports the livelihood of about six million people in Kenya (Ong'injo and Olweny 2009). Annually, 20,130 hectares are developed for sugarcane production in Kenya (KESREF, 2011).

Sugarcane growing is commonly done in Western Kenya, with small-scale farmers contributing 90% and the remaining 10% coming from the large-scale farmers and sugar factories (Odenya *et al*., 2009; KNBS, 2005). By 2005, the Kenyan sugar sector was estimated to produce about 490,000 tonnes of processed sugar, against a domestic demand of 600,000 tonnes (Sserunkuma and Kimera, 2005). The country therefore had a sugar deficit of about 110,000 tonnes, the bulk of which it imported from the Common Markets of Eastern and Southern Africa (COMESA) region. In order to increase the sugarcane production several methods have been adopted such as varieties improvement (Ong'injo and Olweny, 2009), government extension services (Abura *et al*., 2012) and agrochemical methods such as use of fertilizers and pesticides.

2.2 Pesticides use in agriculture

Pesticide use in protection of crops from weeds and diseases is steadily increasing, and is indispensable for the conventional labor-extensive farming system. Some of these chemicals that are used to reduce crop losses due to diseases and pest infestations include; fungicides and bactericides to control crop diseases, nematicides for nematodes, insecticides for insects and herbicides for control of weeds. In soil, the pesticides present diverse behavior, including volatilization, biodegradation, transmission to organisms, uptake by plants, chemical degradation, binding to soil, and leaching into groundwater (Ceballos *et al*., 2004). The fate of pesticides is complex and dependent on factors such as the properties of the pesticide, soil properties and environmental conditions among others (Fang *et al*., 2001).

The environmental fate of pesticides is of great concern today due to the problems resulting from use of mobile and persistent compounds, affecting surface waters and groundwater quality (Miglioranza *et al*., 2003).

2.3 Herbicides use in agriculture

Weeds have been a problem in agriculture since about 10,000 BC. They have always represented one of the main limiting factors in crop production. The damage caused by weeds globally is estimated at 13.2% of agriculture production or about $ 75.6 billion per year. Berca (2004) termed this as "Weeds eat the food of about 1 billion inhabitants." Unlike other pests like insects and disease pathogens, which are sporadic in terms of outbreaks, weeds are relatively constant every season and this poses great threat to agriculture. Some of these threats include competition for light, water, nutrients, and antagonistic effects such as parasitism and allelopathy and reduction of crop quality due to contamination (Pacanoski, 2007). Herbicides are one of the crucial factors in the improvement of agriculture production. Herbicides can effectively control weeds, saving labour necessary for weed control practices and at the same time, reduce soil erosion, save energy, increase crop production and reduce the cost of farming. In Western Kenya, where sugarcane farming is practiced on commercial basis both by local farmers and sugarcane industries, the use of herbicides to control weeds in sugarcane farms for improved sugar production is rampant. Some of herbicides used include glyphosate, diuron, ametryn, metribuzin, lasso, hexazinone and 2,4-D. Hexazinone and 2,4-D are among the most commonly used herbicides to control weed by the Nzoia Sugarcane Company limited in their nuclear estates.

2.4 Hexazinone

Hexazinone [3-cyclohexyl-6-dimethylamino-1-methyl-1,3, 5-triazine-2,4-dione] (CAS: 51235-04-2, molecular formular: $C_{12}H_{20}N_4O_2$, molecular weight: 252.1586) (figure 1) is a broad-spectrum triazine herbicide used for the control of weeds in alfalfa, berries, hay, pineapple, sugarcane, lucerne, woodlands, horticultural nurseries, and along roadways, railways and at industrial sites (Wang *et al*., 2005).

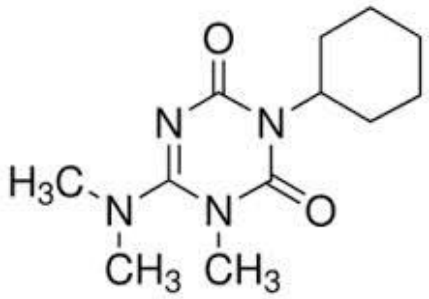

Figure 1. Structure of Hexazinone

Hexazinone (registered with the trade name Velpar^{TM}) is highly soluble in water (33 g L^{-1}) and highly mobile in soil (Jensen and Kimball, 1987). It has a low average organic carbon adsorption coefficient (K_{oc} = 610) and a low octanol/water coefficient (K_{ow} = 15.0) (Mandelbaum and Wackett, 2008). Therefore, it is mobile in the environment with a great potential for leaching and partitions into water more than to soil, or biota. With the moderate to long half-life and high mobility, hexazinone can potentially move offsite with water in run-off and in base flow. The average half-life for hexazinone has been reported as 90 days (Tu *et al*., 2001; Wang *et al*., 2006; Hunter and Shaner, 2012).

2.4.1 Mode of action of hexazinone

Hexazinone works by binding a protein of the photosystem II complex, which in turn blocks the photosynthetic electron-transport chain. This results in a chain of reactions in which triplet-state chlorophyll reacts with molecular oxygen (O_2) to form singlet oxygen (O). Chlorophyll and O strip hydrogen (H^+) from unsaturated lipids in both the cell and the organelle membranes, to produce free radicals. These lipid radicals attack and oxidize other lipids and proteins, causing the cell and organelle membrane to leak. The leakage of the cellular contents leads to cell death and eventually the death of the plant (Perkins, 2002).

2.4.2 Toxicity of hexazinone

Hexazinone exhibits low toxicity to birds and mammals. There are no cases of chronic toxicity associated with hexazinone to human, but most of its effects are acute, such as irritation of eyes, nose, and throat (Hunter and Shaner, 2012). Study on animals shows that the compound is quickly excreted by animal systems. There is little chance that the herbicides bioaccumulates in the tissues of any mammal, including humans (FDA, 1986; USDA, 1994).

Since the mode of action of hexazinone is based on inhibition of photosynthesis its effects may be exerted at the base of the food chain especially on marine ecosystem. Several studies have been conducted to identify negative impacts that the compound might have on

other plants or animals found in lakes, streams, and river habitats. Examination of lakes in boreal forests of Ontario, Canada revealed a depression of phytoplankton at hexazinone concentrations as low as 0.01 mg L^{-1}. These workers also noted that chronic exposure to levels of 0.1 mg L^{-1} caused irreversible damage to the plankton (Thompson *et al*., 1993a). A more extensive study in the same geographical region noted similar declines in zooplankton numbers and concluded that the population change was a result of food resources lost with the suppression of phytoplankton (Thompson *et al*., 1993b; Baillie *et al*., 2015)). Due to the harmful effects of hexazinone and other chemicals there is need to look for ways to eradicate such chemicals from the environment and one way is to explore the use of microflora in the soil since some have been shown to completely degrade or transform xenobiotics including pesticides into non-harmful forms (Mishra *et al*., 2001).

2.4.3 Environmental fate of hexazinone

Hexazinone is highly susceptible to transfer especially through leaching and runoff due to its high-water solubility. This property confers its high potential of contaminating ground water as well as surface water (Wang *et al*., 2006). The environmental fate of hexazinone is primarily attributed to biodegradation by indigenous microorganisms which help in its' clean up from soil. Processes such as hydrolysis, photo-degradation and chemical degradation have shown insignificant effects on hexazinone thus their dependence to remove this compound from the environment may imply persistent residue activity on soil. Hexazinone biodegradation by microorganism in soil involves demethylation and hydroxylation of the cyclohexyl ring and account for the highest mode of hexazinone removal from soil (Ngigi *et al*., 2014; Wang *et al*, 2006).

2.4.4 Microbial degradation of hexazinone

The biodegradation of herbicides is based on the ability of microorganisms to utilize the herbicides in order to derive energy. Hexazinone can serve as sole source of carbon (C) and nitrogen (N). The triazine ring is rich in nitrogen and poor in carbon; this implies that the compound can be used as both carbon and nitrogen source for biodegraders isolation (Hunter and Shaner, 2012). Hexazinone degrading bacteria such as *Pseudomonas sp* and *Enterobacter cloacae* have been isolated from sugarcane soils (Xuedong *et al*., 2003; Wang *et al*. 2005). A similar study by Ngigi *et al*. (2014) isolated *Enterobacter cloacae* associated with hexazinone degradation. Another study that confirmed microbial degradation of hexazinone is that of

Mostafa and Heilling (2003), that reported the degraders *Microbacterium foliorum*, *Paenibacillus illinoisensis* and *Rhodococcus equias*.

2.5. 2,4-Dichlorophenoxyacetic acid as an herbicide

2, 4-D, a member of the chlorophenoxy family of herbicides (CAS: 94-75-7, chemical formula: $C_8H_6Cl_2O_3$; molecular weight: 221) (Figure 2) was the first successful selective herbicide to be developed (Wang and LemLey, 2001). 2,4-D was introduced in 1946 and rapidly became the most widely used herbicide in the world (Wang and LemLey, 2001). Registered forms of 2,4-D include 2,4-D acid, 2,4-D dimethylamine salt (DMAS), 2,4-D isopropyl acid (IPA), 2,4-D triisopropyl acid (TIPA), 2,4-Diethylhexyl ester (EHE), 2,4-D butoxy ethyl ester (BEE), 2,4-D diethyl amine (DEA), 2,4-D isopropyl ester (IPE), and 2,4-D sodium salt. 2,4-dichlorophenoxyacetic acid (2,4-D) (2) is a moderately persistent chemical with a half-life ranging between 20-200 days (Bukowska, 2006).

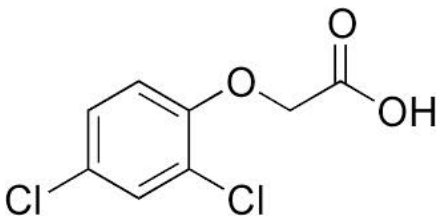

Figure 2. Structure of 2,4-Dichlorophenoxyacetic acid

2,4-D has pKa of 2.6 and high-water solubility (45 g L^{-1}). It presents a systemic mode of action and has been widely employed in wheat, rice, corn, sorghum and sugarcane farms to control harmful wide-leaf weeds (Thill, 2003).

2.5.1 Mode of action of 2,4-D

2,4-D and other members of chlorophenoxy herbicides mimic the mode of action of growth hormone auxin in plants (Grossmann, 2000). When the herbicide is present in low concentrations at the cellular site of action, growth by cell division and elongation is usually stimulated. However, with increasing concentrations, varieties of abnormalities are induced within 24 hours of treatment (Grossmann, 2000). These include curvature of leaves and stem, growth inhibition of shoot and root, and intensified leaf pigmentation. A number of biochemical reactions are involved in the development of these abnormalities in the plants, for example, overproduction of ethylene, leading to stomata closure and thereby carbon

assimilation inhibition. Eventually, the exposure results in desiccation, necrosis and plant death (Song, 2014). 2,4-D is a selective herbicide that destroys dicot plants. Monocots are insensitive to it, because they eliminate the herbicide by degradation. For this reason, 2,4-D is used for combating weeds in cereal crops.

2.5.2 Health effects of 2,4-D

2,4-D is classified by both Brazilian National Agency for Sanitarian Vigilance (ANVISA) and World Health Organization (WHO) as a hormonal herbicide of level II toxicity. It is considered a carcinogenic agent, affecting liver, heart and central nervous system, leading to convulsions (Garcia *et al*., 2006). In mammals 2,4-D disrupts energy production depleting the body of its primary energy molecule, adenosine triphosphate (ATP) (Palmiera *et al*., 1994). Its carcinogenicity is attributed to dioxins or polychlorinated dibenzo-dioxin (PCDDs), a group of chemicals known to be hazardous to human health and to the environment (Littorin, 1994) Numerous epidemiological studies have linked 2,4-D to non-Hodgkin's lymphoma (NHL) among farmers (Morrison, 1992; Zahm, 1997; McDuffie *et al*., 2001). The teratogenic, neurotoxic, immunosuppressive, cytotoxic and hepatoxic effects of 2,4-D have been well documented (Tuschl and Schwab, 2003).

A study by Rawlings *et al*. (1998) showed that 2,4-D caused significant suppression of thyroid hormone levels in ewes dosed with this chemical. Similar findings were reported in rodents, and decreases in weight of the ovaries and testes. In rodents, this chemical also increases levels of the hormones progesterone and prolactin, and causes abnormalities in the estrus cycle (Duffard *et al*., 1995; Charles *et al*., 2001). Thyroid hormone is known to play a critical role in the development of the brain. Slight decline in thyroid hormone show adverse effects in neurological development in fetus, resulting in lasting effects on child learning and behavior (Haddow *et al*., 1999). 2,4-D has negative effects on reproduction hormones (Liu, Hahn and Hurtt, 1996). A research by Lerda and Rizzi, (1991) also indicated that male farm sprayers exposed to 2,4-D had lower sperm counts and more spermatic abnormalities compared to men who were not exposed to this chemical.

2.5.3 Environmental fate of 2,4-D

2,4-D is highly soluble in water and this makes the compound have high transfer rate as mentioned for hexazinone (Waite *et al*., 2002). Due it is chemical structure, 2,4-D is somewhat resistant to hydrolysis though as the pH of soil increase, the chemical may become more hydrophilic, especially at pH greater than 8. The chemical is also resistant to photo-degradation process (Bukowska, 2006). However, although the chemical structure of 2,4-D

seems complex, it is readily degraded and used as a carbon source by various environmental microorganisms. And several researches have suggested microbial biodegradation of 2,4-D as the best remedy for its removal from contaminated soils (Silva *et al.,* 2007).

2.5.4 Microbial degradation of 2,4-D

There are several strains reported as 2,4-D degraders using at least two main different pathways, *α-Ketoglutarate* or *dehydrogenase* pathways as shown in figure.3 (Kitagawa *et al.,* 2002). Van der Meer (1994) suggested that metabolic activity towards xenobiotics such as 2,4-D may have emerged from altered enzymatic specificity of existing enzymatic systems, which are effective for the biodegradation of naturally occurring aromatic compounds. On the other hand, evidence of independently acquired genes indicate an active selective process in the evolution of this complex 2,4-D biodegradation pathway (Dejonghe *et al*., 2000). There are several bacteria that have been identified to have the potential of 2,4-D degradation. These includes; *Acinetobacter* sp. *Serratia marcescens, Stenothrophomonas maltophilia* and *Flavobacterium* sp. (Silva *et al*., 2007). All these bacterial strains have capsules and are Gram negative. Other studies have shown *Ralstonia eutropha* formerly known as *Alcalgenes eutrophus* to have potential for 2,4-D degradation using plasmid pJP4 as shown in figure 3 (Hotopp and Hausenger, 2001). Study by Balajee and Mahadevan (1990) also revealed *Azotobacter chroococcum* ability to degrade 2,4-D to 3,5-dichlorocatechol which is then degraded to the intermediate of general metabolism.

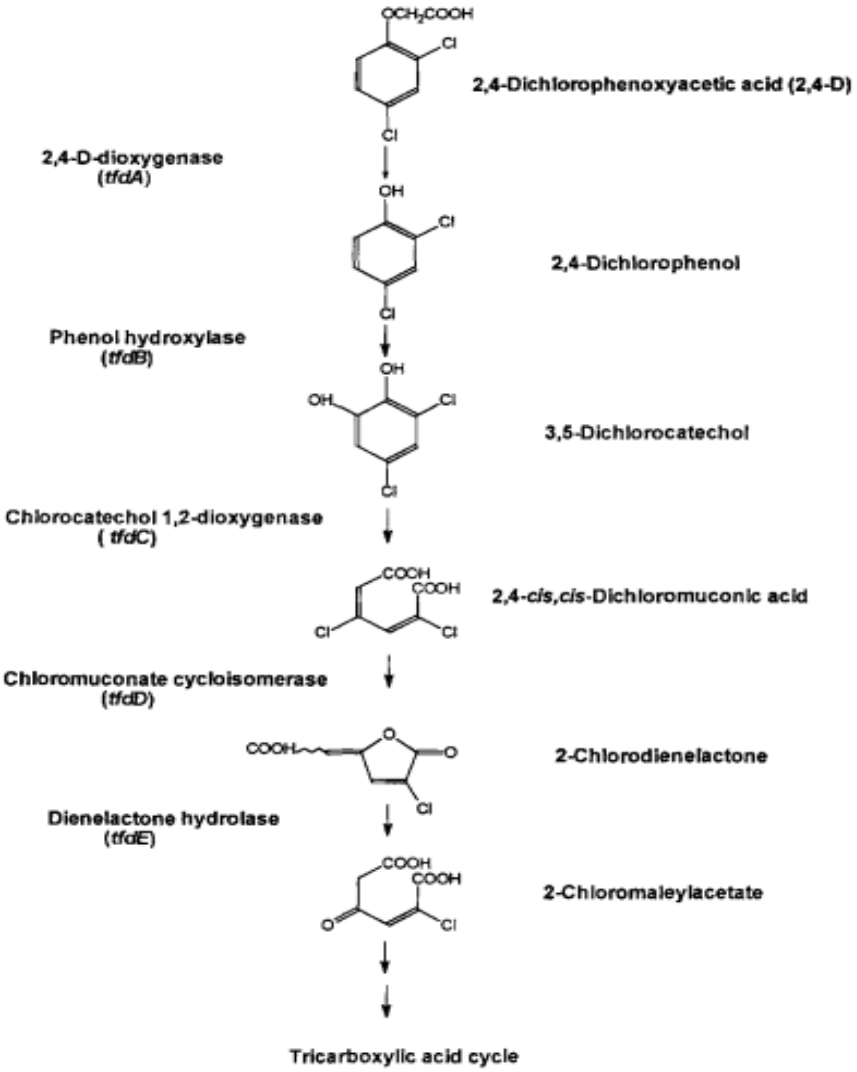

Figure 3. 2,4-D degradation pathway in *A. eutrophus*

Even though several studies have been done to isolate and identify the biodegraders of hexazinone and 2,4-dichlorophenoxyacetic acid, this field is still unexhausted. Microbial community is characterized by continual mutation, which help in adaption to the changing environmental conditions (Denamur and Matic, 2006), for example those caused by some of xenobiotics used in agricultural practices like hexazinone and 2,4-D. Moreover, studies have revealed that the degrading ability of microorganism towards organic compounds defer depending on the environmental factors such as pH, temperature, nature and moisture of the soil (Kah *et al.,* 2007). This brings out the necessity to investigate the types of bacteria involved in biodegradation of these compounds wherever they are applied. This study aimed at isolating and characterizing native bacteria with capability to degrade hexazinone and 2,4-D in Nzoia Sugar Company sugar cane farms.

2.6 Dehydrogenase enzymes as bioindicator of soil microbial activities

Microbial communities are vital for the normal operation of the ecosystem in relation to direct interaction with fauna and in nutrients and organic matter cycling. Enzymes, which are part of the microbial cellular metabolism, are important in the life processes and therefore play significant roles in maintaining soil health and its environment. Soil enzymes are a group of enzymes, which are found in soil and play a crucial role in maintaining soil ecology, physical and chemical properties, fertility and soil health (Adak *et al*., 2014; Das and Varma, 2011). The enzymatic activities in the soil are majorly of microbial community origin being derived from intracellular, cell-associated or free enzymes. The enzymes act as mediators and catalysts of important soil functions that include: decomposition of organic inputs; transformation of native soil organic matter; release of inorganic nutrients for plant growth; N_2 fixation; nitrification; denitrification; and detoxification of xenobiotics. In addition, soil enzymes have a crucial role in C (β-glucosidase and β-galactosidase), N (urease), P (phosphatase), and S (sulphatase) cycle (Martinez *et al*., 2010).

The amounts of the enzymes in soils vary due to varying amount soil of organic matter content, different composition and activity of the living organisms and varying intensity of biological processes (Fontaine *et al*., 2003). Analysis of enzymes in soil provides essential information on biological processes taking place. Enzymes in the soil are sensitive to both anthropogenic and natural interferences and they can be used to elucidate any induced changes in soil ecosystem (Kizilkaya and Aşkin, 2007). For instance, some researches have revealed high dehydrogenase (DHA) activities in soils collected from forests where there are less anthropogenic disturbances compared to farms areas where there are frequent management activities (Kumar *et al*., 2013). Examples of the enzymes found in the soil include; amylase, arylsulphatases, β-glucosidase, cellulose, chitinase, DHA, phosphatase, protease, and urease released from plants (Das and Varma, 2011).

Soil DHA enzymes are among the key enzymes involved in soil biochemical processes and maintaining soil biogeochemical cycles. DHA enzymes belong to the oxidoreductases (EC 1.1.1) class of enzymes and catalyze the oxidation of organic compounds by separating Two-H atoms. The separated H atom is mostly transferred to nicotinamine adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (Wolińska and Stępniewska, 2012; Kumar *et al.*, 2013). Measurement of soil DHA enzymes activity was initiated by (Lenhard, 1956). DHA activity measurement on soil has been used for decades and can be considered as the most important and sensitive indicator of soil microbial activity (Cycoń *et al*., 2010; Järvan *et al*., 2014). This is owing to the fact that,

unlike other enzymes in soil, DHAs are intracellular enzymes and occur in all viable microbial cells. The intracellular nature of the enzymes makes it a good parameter for the analysis of the viable cells in the soil. In addition, DHA enzymes are immediately degraded following cell death and therefore their detection is only in the living cells (Kizilkaya and Aşkin, 2007). Its analysis gives a good correlation between biological activity in the soil and the microbial population at time of analysis (Kumar *et al.*, 2013). Measurement of DHA enzymes is based on the redox reaction process in which 2, 3, 5- triphenyltetrazolium chloride (TTC) is reduced to the creaming red-colored triphenylformazan (TPF). TTC acts as an electron acceptor in anaerobic soil environment condition during the electron transport chain (ETC) process of microbial origin. Subsequently TTC which is water soluble and colorless is reduced by microbial DHA enzymes to formazan which is a red color water insoluble dye and can be quantified calorimetrically by visible light at 485nm (Wolińska and Stępniewska, 2012; Mambu, 2014).

Several studies have been conducted on the effects of hexazinone and 2,4-D on other diversity of life. However, there is limited information on the impacts that these herbicides may have on the soil microbial community. Therefore, this determined the variation of microbial activity upon application 2,4-D and hexazinone using DHA as the indicator tool and their effects on soil microbial biomass. The study also isolated the bacterial degraders of the two herbicides, determined the biodegradation of the two herbicides using the isolated bacteria and characterized the isolates through biochemical and molecular methods.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The study was conducted in Nzoia Sugar Company farms in Bungoma County, Kenya. The sugar plantations have many water canals, which drain into Kuywa River that traverses through the farms and is one of the Nzoia river tributaries. Nzoia River itself originates from Cherangani Hills at a mean elevation of 2,300 m above sea level (asl) and drains into Lake Victoria at an altitude of 1000 m asl. Nzoia River Basin lies between latitudes 1º 30′N and 0º 05′S and longitudes 34º and 35º 45′E. It runs approximately South-West and measures about 334 km with a catchment area of about 12,900 km^2, with a mean annual discharge of 1777 x 10^6 m^3/year. River Nzoia basin hosts more than 3 million people and a good proportion of the population work as peasant farmers in the sugar industry. The river is of regional importance as it contributes enormously to the shared waters of Lake Victoria. Many other rivers feed the Nzoia before it discharges into Lake Victoria.

Soils for the study were obtained from five selected nucleus estate sugarcane farms (139, 212, 242, 13010 and 15030) of Nzoia Sugar Company Limited (which lies between 34^{o}50′49" E-35^{o}35′41" E longitudes and 0^{o}4′55" N-0^{o}20’11" S latitudes), in which 2,4-D and hexazinone herbicides had been applied consecutively from the year 2012, and from a nearby out grower farm (OGF) in Western Kenya–Bungoma County (Figure 4).

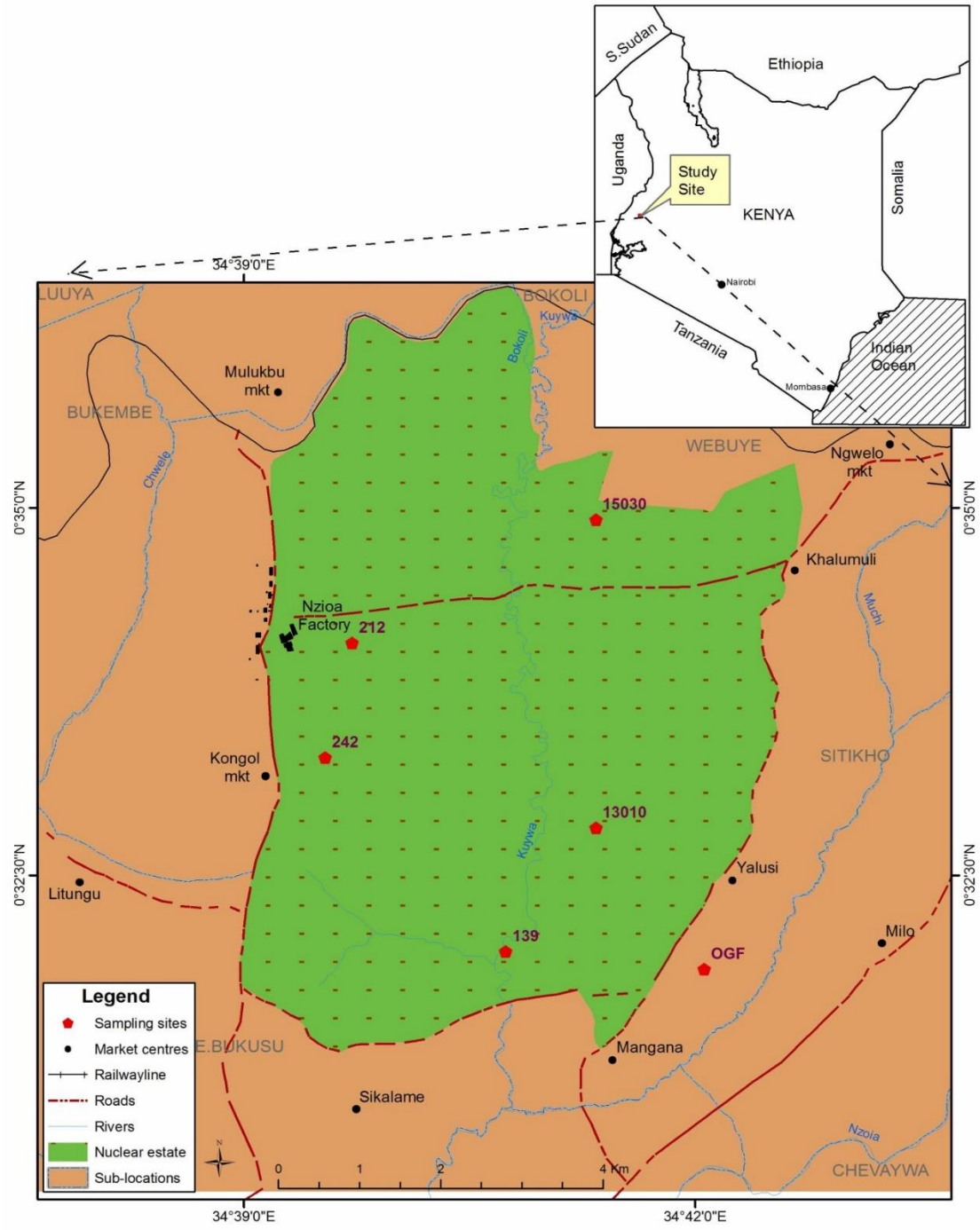

Figure 4. Geographical location of Nzoia Sugar Company farms in Bungoma County (courtesy of Mr. Geoffrey Maina, Cartographer, Department of Environmental Science, Egerton University, 2016).

3.2 Soil sampling and pre-preparation of soil samples

Soil sampling was done using a soil auger to obtain soil samples at a depth of 0-15 cm. The sampling was done randomly from at least three sites within the specified farms (Figure 5). Soil temperature was taken during sampling using a portable handled thermometer (Digital LCD IR Infrared Laser Gun). The samples were carried to the laboratory using cool

box and stored at 4 ^{o}C upon arrival to the laboratory at Egerton University where analysis was performed. The sub-samples were dried and passed through <2 mm diameter sieve. These sub-samples were homogenized and mixed to make a composite sample for each farm. The samples were kept at 4 ^{o}C for subsequent analysis.

(a) (b) (c)

Figure 5. (a) Sugarcane plantation in Nzoia Sugarcane Company Limited (b) Soil sample collection using soil auger 0-15 cm, (c) taking of soil temperature using a portable thermometer handheld Digital LCD IR Infrared Laser Gun

3.3. Chemicals and Reagents

Hexazinone (99%), 2,4-D (99%) and 3,5-DCC (99%) were purchased from Sigma Aldrich, Germany through Kobian Company Ltd (Nairobi, Kenya). All other chemicals were of analytical grade and purchased from Kobian Company Ltd (Nairobi, Kenya). HPLC-grade solvents for the study were purchased from the same company.

3.4 Determination of physicochemical characteristics of soils

Soil temperature was measured and recorded in the field, during sampling, using a laser thermometer, Raytek®RAYRPM30L2G, USA). The pH of the soil was measured in a soil water suspension (soil: water ratio of 1:2), by standard method described by Geotechnical Engineering Bureau (2007). 30 g of soil was weighed and put in a glass beaker and 30 mL of distilled water added to the sample and stirred. The sample was let to stand for one hour with stirring every 10 -15 minutes to allow pH of the soil slurry to stabilize. The temperature reading of the pH meter was adjusted to that of the sample, before testing. The pH meter was standardized by means of buffers of pH 4.0, 7.0 and 10.

Soil moisture content was measured using the method described by Black (1965). 10 g of soil was weighed in aluminium tin and dried overnight in an oven at 105 ^{o}C. The weight of the dry soil sample was then recorded. The soil sample was returned in the oven, and dried further at the same temperature until no difference between any two consecutive weight measurements was recorded. The moisture content was calculated as the difference in the soil weight after drying and expressed in percentage.

The amount of Nitrogen (%) was analyzed using Kjeldahl methods (Anderson and InGram, 1994) and measured using FOSS TECATOR digester machine (2200 Kjeltec Auto Distillation, Sweden). The soil sample was air dried at room temperature (25 ^{o}C), ground and sieved using 2 mm sieve. A sample of 0.3 g of air dried soil was weighed into a test tube and 4.3 mL of digesting solution (selenium powder, hydrogen peroxide, H_2SO_4 and lithium sulphate) added. The test tubes were put in digestion block for 3 hours set at 360 ^{o}C to obtain clear solution (indicate complete digestion). The samples were allowed to cool at 25 ^{o}C for 30 minutes after which they were diluted to 100 mL using 46% NaOH into a conical flask. 10 mL aliquots of the samples and NaOH were taken and distilled. A recipient (1% boric) was prepared. 5 mL of the recipient was mixed with the sample and titrated against standardized acid (0.01 normal HCL) until a pink colour was noted. The volume of the titres were recorded and the %N given as;

$$\%\, Nitrogen\ = \frac{(\mathrm{a}-\mathrm{b})\mathrm{N}\times 0.014\times 100\times 100\prime}{\mathrm{a}\prime\times 0.3} \quad (1)$$

Where a is volume of sample titre, b is black titre, N is normality of the acid used (0.01 N HCL), 0.014 is molecular weight of N in a litre (1000 mL), 100 is the dilution factor, 100′ is conversion factor into percentage and a′ is mL of aliquot taken for analysis.

In order to analyze potassium, calcium and magnesium, the samples were prepared similar to those for nitrogen but no distillation was done. The amounts of the elements were determined using atomic absorption spectroscopy (210 VGF AAS, USA). The quantification was done using calibration curves prepared using known amounts of standards compounds for K, Ca and Mg.

Phosphorous was analyzed using Mehlich method (Horneck *et al*., 2011). Thus, 5 g of air dried soil was weighed into 250 mL conical flasks. 50 mL of extracting solution (H_2SO_4 and HCL) was added into the samples. Free activated charcoal was then added and the samples put in an electric shaker for 30 minutes. After shaking, the samples were allowed to settle for 10 minutes and then filtered and the filtrate collected. 5 mL of the filtrate was sampled for colour development using colour developer (vanadium and molybdate in the

ratio 1:1). The colour was allowed to develop for 30 minutes and the measurement done using spectrophotometer (UV-200-RS, USA) at 430 nm wavelength. The quantification was done using calibration curves prepared using known amounts of P standard compound.

3.5 Determination of the effects of 2,4-D and hexazinone on soil microorganisms and DHA activity

3.5.1 Assessment of inhibition of 2,4-D and hexazinone to soil microorganisms

In order to confirm whether there was any toxicity of the two xenobiotics to the soil microorganisms, colony forming unit (CFU) experiments were determined according to the method described by Curtis *et al.* (2002). To this end, fresh soil from a farm which had never received the two herbicides (OGF) for the control of weeds was used. The samples were sieved through < 2 mm sieve to remove any unwanted debris and to homogenize the soil. The samples were then put on an aluminum foil at room temperature in three sub-sets. Two of the sub-sets were treated with 2,4-D and hexazinone at the field recommended rate and one of the sub-sets was used as the control. The samples were treated with water to appropriate moisture content (60% water holding capacity). The number of colony forming units in the soil samples was determined using serial dilution technique and the pour plate method. For enumeration of the bacteria, 1 g of soil sample was put in 9 mL distilled water and serially diluted to $\times 10^{-3}$. In order to enumerate the bacteria, 1 mL of the $\times 10^{-3}$ was then poured on sterile petri dish in which 15 mL of sterile molten nutrient agar was poured and the plates incubated at 30 ± 1 ^{o}C. The effects of the herbicides on viable bacteria counts were monitored in the soils each day for seven days.

3.5.2 Determination of DHA activity

The DHA activity was determined according to the method described by Lenhard (1956), based on the reduction of 2,3,5-triphenyltetrazoliumchloride (TTC) (Aldrich, Germany) to the creaming red colored formazan (TPF). In this study, 6 g of homogenized soil sample from each farm was weighed into test tubes in triplicates. 1 mL of 50 μgmL^{-1} (field recommended application rate) hexazinone and 2,4-D were prepared and used to treat the samples in triplicates. 1 mL of 3% TTC solution was added to each sample followed by an addition 2 mL of deionized distilled water. The test tubes were caped tightly to exclude the air since the activity of TTC is greatly affected by oxygen. The samples were incubated in dark at 30 (± 1) ^{o}C for seven days with analysis of the TPF at each day. To the control replicates, 1 mL of 3% TTC was added with no addition of the two herbicides and subjected

to the same conditions. The hydrolytic product of the TTC, TPF, was extracted by passing 50 mL analytical grade methanol through 6 g of the sample held on 4 µm cellulose glass microfilter paper on a vacuum pump until all the red color was completely collected (Figure 6). The collected formazan (red color) was analyzed at 485 nm using a scanning spectrophotometer (Genesys 10-S 10 UV, Thermo Fisher Scientific, Austria) and the amount of the TPF expressed as µg TPF/g soil sample. The concentration of the TPF is a direct representation of the DHA activity (redox processes) taking place in respective soil samples. The data was presented as means ±Std.Dev of triplicates samples for each farm.

In order to quantify the amount of TPF present in the samples, a standard curve was prepared using standard formazan (Aldrich, Germany). Varying concentration of 0, 5, 10, 15, 20, 25 and 30 μgmL^{-1} were prepared in analytical grade methanol. From these concentrations an aliquot of 1 mL was used to analyze the absorbance. The absorbance (A) readings versus the concentrations of the standards formazan were used to plot a standard curve from which the formazan concentrations in samples were quantified.

Figure 6. (a) Vacuum pump extraction of formazan from soil sample, (b) an extract of formazan from soil sample ready for spectrophotometric analysis.

3.6 Extraction of hexazinone, 2,4-D and residues 3,5-DCC from soil

The extraction was carried out by soxhlet method. A 10 g sub-sample of the homogenous air-dried soil was weighed, put inside 50-mL cellulose thimble and extracted continuously with 25 mL HPLC-grade methanol (Bicalho *et al*., 2010). The whole extract of

25 mL was dried by use of anhydrous sodium sulfate and filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters. The eluate was then concentrated to a volume of 1 mL with a rotary evaporator.

3.7 Isolation of hexazinone and 2,4-D degrading bacteria

3.7.1 Inocula preparation and culturing

In order to select the herbicides' tolerant bacteria, selective enrichment with mineral salt medium (MSM) method was used (Newby *et al*., 2000; Mostafa and Helling, 2003). Mineral salt medium (g l^{-1}) [3.5 g K_2HPO_4; 1.5 g KH_2PO_4; 0.3 g $MgSO_4.7H_2O$; 0.03 g $Fe_2(SO_4)_3.9H_2O$; 0.03 g $CaCl_2.2H_2O$] with an addition of, 0.01g MoO_4^{-2}, 0.01g $CoCl_2$ 0.03 g, $(NH_4)_2SO_4$ and 0.03 g (NH_4NO_3) for isolation of 2,4-D degrading bacteria, was prepared and sterilized. Iron sulfate was filter-sterilized and added to the medium after autoclaving to avoid formation of precipitates. Aliquots of 49 mL of the sterile MSM were amended with 50 mgL^{-1} hexazinone and 60 mgL^{-1} 2,4-D, respectively. An aliquot of 1 g of soil was suspended in 9 mL of 0.9% NaCl in distilled sterile water, vortexed for 2 min and diluted to 10^{-3} in ten-fold dilution steps. Dilutions of the soil samples (aliquot of 1 mL) were used for inoculating the 49 mL MSM with 50 mgL^{-1} hexazinone as the sole source of carbon and nitrogen and 60 mgL^{-1} 2,4-D as the sole source of carbon in 250 mL -Erlenmeyer flask. The liquid cultures were incubated (in triplicates) at 30 ± 1 ^oC with shaking at 120 rpm in a darkened thermostatic orbital shaker for a period of 72 hours. After this period, 1 mL of the culture was transferred to freshly prepared 49 mL MSM supplemented with 50 mgL^{-1} hexazinone and 60 mgL^{-1} 2,4-D and incubated at the same conditions for an additional 72 hours. After five subsequent transfers in fresh MSM, serial dilutions were made from the liquid MSM and plated to a solid agar supplemented with 50 mgL^{-1} hexazinone and 60 mgL^{-1} 2,4-D in order to isolate individual colonies. For the isolation and purification of the pure distinct colonies repeated sub-culturing on the same solid MSM medium was done five times. Four strains capable of growing in hexazinone amended MSM were isolated and designated as N13010H1, N15030H2, N15030H3, and N212H4 and three isolates capable of growing in 2,4-D amended MSM coded as N139D1, N13010D3, and N13010D4 and used for further analysis.

3.7.2 Determination of individual bacteria degradation rate

In order to carry out biodegradation rate kinetics of the isolated bacteria, bacteria cell suspension at a concentration of approximately 6×10^8 cells mL^{-1} were prepared using 0.5

McFarland solutions. 1 mL of every single bacteria strain cell suspension was used to inoculate 49 mL of MSM amended with 50 mgL^{-1} hexazinone as the only sole source of carbon and nitrogen and 60 mgL^{-1} 2,4-D as the only source of carbon in 250 mL-Erlenmeyer flasks. MSM+bacteria inoculum and without 2,4-D or hexazinone were used as control. The cultures were incubated at 30 ± 1 ^{o}C with shaking at 120 rpm in a darkened thermostatic orbital shaker for a period of 28 days. To determine the growth rate of these hexazinone and 2,4-D-degrading bacteria strains, 2 mL of the MSM was sampled aseptically at every 48 hours interval. The growth of the bacteria was determined spectrophotometrically by measuring the optical density (OD) at 600 nm. The same aliquots were preserved at 4 ^{o}C and used later for HPLC analysis.

Degradation kinetics for the two herbicides was fitted to first-order kinetics, hence described by the following equations:

$dC/dt = -Kt$…… i

$LnC = -Kt + lnC_o$…… ii

$t_{1/2} = 0.693/K$……. iii

Where C is concentration of pesticide remaining in soil (mg mL^{-1}), K is degradation rate (mg mL^{-1} day^{-1}), C_o is the initial concentration of pesticide in soil (mg mL^{-1}), $t_{1/2}$ is the half-life (days) (Wang *et al*., 2005; Bandala *et al*., 2007).

3.7.3 Preparation of samples for HPLC analysis of hexazinone and 2,4-D.

The cleaning of samples for HPLC analysis was done according to the method described by Bicalho *et al*. (2010) in which liquid-liquid extraction method was used. 2 mL of ethylacetate was added to 1 mL of the MSM-bacterial sample and vortexed for 1 minute. The sample was allowed to settle for the separation of the aqueous and organic phase. Using a pipette, the top layer of the aqueous phase was siphoned and put in a separated test tube. The organic solvent was then evaporated in rotary evaporator and the residues reconstituted using 1 mL HPLC-grade methanol and kept at 4 ^{o}C prior to HPLC analysis.

3.8 Instrumental analysis of herbicides and metabolite residues

3.8.1 Preparation of standard calibration curves

In order to quantify the amount of the herbicides residues present in the soil and the degrading ability of bacteria in the MSM media, standard calibration curves for the compounds were prepared. For hexazinone eight calibration dilutions of varying concentration were prepared from the standard stock solution using HPLC- grade hexazinone

as follows; 0, 2.5, 5, 10, 20, 30, 40 and 50 mg L^{-1} in HPLC-grade methanol. For 2,4-D and 3,5-DCC, the concentrations were 0, 2.5, 5, 10, 20, 30, 50 and 60 mg L^{-1}.

3.8.2 HPLC analysis of herbicides and metabolite residues

HPLC analysis was performed using Shimadzu LD (CBM-20A, Japan) equipped with a UV-VIS detector (ProStar 325), solvent delivery module (ProStar 210), and reverse-phase column (Discovery® HS C18, 25 mm × 4.6 mm × 5 µm)). For chemical detection, the mobile phase was acetonitrile: water (60:40, v/v), injection volume 20 µl at a flow rate of 1.0 $mLmin^{-1}$ and detected at 254 nm for hexazinone and 225 nm for 2,4-D and 3,5-DCC. The retention time under these chromatographic conditions was 5.4 minutes for hexazinone, 7.5 minutes for 2,4-D and 8.1 minutes for 3,5-DCC. The data obtained was analyzed using chromatography workstation software (LC Real Time Analysis) and later transferred to excel. A linear regression of the peak areas versus the concentration of the standard compounds were obtained and used in quantification of herbicide residues and metabolite 3,5-DCC in soil extracts and in mineral salt medium.

3.9 Morphological and Biochemical characterization of bacterial isolates

Morphological and biochemical characterization was done according to the Bergys Manual of Systematic Bacteriology (Goodfellow *et al*., 2012). Biochemical tests done on the isolates included; gelatin hydrolysis, starch hydrolysis, catalase test, indole test and Gram staining.

I. Indole test

The test bacterial isolates colony was aseptically inoculated into tryptophan broth using a wire loop in test tubes and incubated at 37 ^{o}C for 48 hours. After incubation, 0.5 mL of Kovacs reagent was added to the broth culture and shaken gently to observe any colour change and red ring formation.

II. Gelatin hydrolysis

The test bacterial isolates were inoculated aseptically using wire loop (by stabbing) into nutrient gelatin medium [in gL^{-1} 120 gelatine, 3 meat extract, and 5 peptone; pH 6.8±0.2 (20 °C) in 1000 mL distilled water]. The inoculums were incubated at 37^{o}C for two weeks. The cultures were checked after 2 weeks for gelatin liquefaction by placing the culture tubes in ice for at least 15 min or until the control liquefies. Those tubes which did not liquefy were negative while those that liquefied were positive for gelatinase.

III. Catalase test

The test bacterial isolates were tested for catalase enzymes production by placing a small amount of inocula onto a slide and a few drops of 3% hydrogen peroxide added and mixed. Rapid and elaborate bubbles of oxygen indicate that the organism was catalase positive while lack of catalase activity was evident by lack or very weak bubble production.

IV. Starch hydrolysis

The test bacterial isolates were aseptically inoculated using wire loop sterile starch agar media [meat extract 3.0 gL^{-1}, peptic digest of animal tissue 5.0 gL^{-1}, starch soluble 2.0 gL^{-1}, agar 15.0 gL^{-1}, pH 7.2±0.1 (25°C)] and incubated at 37 ^{o}C for 24 h. After incubation the media plates were flooded with iodine reagent. Iodine produces blue coloration in presence of starch. Blue colour development implied that the test organism was negative (-ve) for amylase enzyme while yellow colour development meant the organism is positive for amylase production.

V. Gram staining

A smear of the bacteria colony was made on a glass slide using sterile wire loop and heat fixed. The primary stain Crystal violet (CV) was applied to the heat fixed smear of the bacterial culture. A mordant, in this case Gram's iodine was applied and the decolorization process was done using 95% ethyl alcohol. Finally, a counterstain was done using Safranin to differentiate Gram positive from Gram negative bacteria. The slide was observed on the microscope under the high/oil power magnification lens for blue-black/purple colonies (Gram positive) and pinkish (Gram negative) colonies.

3.10 Molecular characterization

Those isolates that showed positive results for herbicide degradation were subjected to molecular analysis. This involved extraction of DNA, PCR and sequencing of PCR products. The steps are described in details in the following sections.

3.10.1 Bacterial DNA extraction

Total microbial DNA isolation was done at the Institute of Biotechnology Research at Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya. The total microbial DNA was isolated using method described by Schmidt *et al*. (1991). Two known bacterial samples *E. coli* (ATCC 25922) and *B. subtilis spizizenii (ATCC 6633)* purchased through DLD Scientific, (Durban North, South Africa) were used as the positive control standards. Total microbial DNA was extracted from the colonies that had been grown

overnight in nutrient agar media. Bacterial cells were emulsified in 250 µL lysis buffer (0.1 M NaCl, 50 mM disodium EDTA, 0.1 M Tris-HCl, pH 8) containing 0.5% (w/v) sodium dodecyl sulfate 0.5% (w/v), proteinase K (0.5 $mgmL^{-1}$), and RNase (0.8 $mgmL^{-1}$). After emulsification, the tube was placed in a water bath at 60 °C for 5 minutes. Two hundred micro-litres of the supernatant were placed in a new Eppendorf tube and over layered with 30 µL of 6 M NaCl and 2 volumes of room temperature 70% ethanol. The tube was kept at room temperature for 5 minutes, centrifuged at 1000 X g for three minutes. The supernatant was discarded and crude DNA rinsed with 70% ethanol and dried out by leaving the Eppendorf tubes open for a few minutes in the clean bench. Finally, the DNA was suspended in 200 µL of sterile water. The DNA was detected by evaluating its concentration (an OD of 1 at 260 nm = 50 μgml^{-1} of DNA) and its purity (OD at 260 nm/OD at 280 nm = 1.7-2.0). The total DNA was used as a template for the amplification of 16S rRNA gene.

3.10.2 Amplification of 16S rRNA gene

The 16S rRNA gene sequences were PCR-amplified using universal primers Bac8f, forward, 5′-AGRCTTTGATCCTGGCTCAG-3′and Bac1492r, reverse, 5'-CGGCTACCTTGTTACGACTT - 3' using advanced Primus 96 PCR model. Amplification was done in a 30 µL mixture containing 3 µL of 109 PCR buffer, 4 µL of 2.5 mM dNTPs, 2.5 µL of Bac8f forward primer (5 pmol), 2.5 µL of Bac1492r reverse primer (5 p mol), 0.4 µL of 5 U/ µL Taq polymerase, 1.5 µL template DNA and 16.1 µL of PCR grade water. The negative control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles: initial denaturation at 94 ^{o}C for 5 min, 30 cycles of denaturation at 94 ^{o}C for 45 s, primer annealing at 55 ^{o}C for 50 s, chain extension at 72 ^{o}C for 90 s, and a final extension at 72 ^{o}C for 8 min. The amplicons (5 µL) of each DNA sample, together with 1 Kb ladder was loaded on an ethidium bromide containing agarose gel (1%) in 19 TAE buffer and run at 70 V for 1.5 h. The DNA bands were visualized using DNA UV Doc (Mitsubishi).

The 16S rRNA gene sequencing was done in Macrogen, Inc Korea. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using universal primer (Bac8f and Bac1492r). The fluorescent-labeled fragments were purified from the unincorporated terminators with BigDye® XTerminator™ purification protocol. The samples were re-

suspended in distilled water and subjected to electrophoresis in an ABI 3730 x l sequencer (Applied Biosystems).

The 16S rRNA sequences were compared with the sequences in the public database using the BLAST search proGram in the National Centre for Biotechnology Information (NCBI) to find closely related bacterial 16S rRNA gene sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene sequences of the isolates and those of the closely related bacteria were then aligned using ClustawX2 proGram (Thompson *et al*., 1997). The closely related 16S rRNA gene sequences were then used to generate phylogenetic tree by Maximum Likelihood Method using MEGA6 proGram (http://www.kumarlab.net/publications) (Tamura *et al*, 2013).

3.11 Data analysis

All the data was collected in triplicate and means determined and presented as ± standard deviation. The data obtained were presented as tables or graphs using Microsoft Excel 2010, Ink. The statistical analysis was done using SAS version 9.1 portable for windows. The means values for soil physicochemical parameters, the DHA activity values and bacterial colony forming units were subjected to ANOVA for the means comparison and the means differences ranked using Fisher's least significant difference (LSD) test. The Pearson Correlation was done for the bacterial colony forming units and DHA activity using SAS.

CHAPTER FOUR

RESULTS

4.1 Soil Physicochemical Parameters

The soil physicochemical parameters which includes nitrogen content, phosphorous, potassium, calcium, magnesium, pH, temperature and moisture content for the six farms where soil samples for the experiment were analysed. Table 1 shows physicochemical parameters obtained from the five selected pesticide treated farms and from one out grower farm (OGF).

Table 1: Physical-chemical parameters of soil from the six farms

	Soil Properties										
FARMS	**% N**	**P** mg kg^{-1}	**K** mg kg^{-1}	**Ca** mg kg^{-1}	**Mg** mg kg^{-1}	**pH**	**Temp**	**% Moisture**	**%TOC**	**%WHC**	**Texture**
212	0.38 ± 0.01^{ab}	24.74 ± 0.06^{c}	1.44 ± 0.09^{d}	6.02 ± 0.01^{c}	116.78 ± 0.03^{d}	4.94 ± 0.43^{ab}	21.67 ± 0.33^{c}	20.41 ± 2.58^{ab}	2.51 ± 0.06^{ab}	84.82 ± 0.34^{a}	Clay Loam
139	0.34 ± 0.03^{b}	21.49 ± 0.02^{e}	1.41 ± 0.01^{d}	0.55 ± 0.02^{d}	115.76 ± 0.04^{e}	5.04 ± 0.37^{a}	23.67 ± 0.88^{bc}	22.35 ± 2.75^{a}	2.04 ± 0.72^{ab}	76.02 ± 0.34^{b}	Sandy Clay
242	0.34 ± 0.02^{b}	22.46 ± 0.06^{d}	13.79 ± 1.10^{b}	0.62 ± 0.00^{d}	216.59 ± 0.28^{a}	4.18 ± 0.17^{b}	25.00 ± 1.53^{ab}	16.24 ± 0.58^{b}	1.40 ± 0.37^{b}	41.13 ± 0.78^{e}	Sandy Loam
13010	0.38 ± 0.01^{ab}	144.39 ± 0.32^{a}	13.59 ± 0.18^{b}	46.43 ± 0.38^{b}	121.29 ± 0.13^{c}	5.00 ± 0.21^{a}	27.00 ± 0.58^{a}	21.46 ± 1.75^{ab}	1.72 ± 0.39^{b}	34.50 ± 5.56^{e}	Sandy Loam
15030	0.35 ± 0.01^{b}	21.48 ± 0.03^{e}	10.81 ± 0.10^{c}	6.48 ± 0.26^{c}	101.20 ± 0.14^{f}	5.05 ± 0.10^{a}	22.67 ± 0.33^{c}	17.92 ± 1.69^{ab}	1.38 ± 0.49^{b}	57.41 ± 2.54^{d}	Loam
OGF	0.43 ± 0.01^{a}	31.43 ± 0.47^{b}	44.37 ± 0.45^{a}	67.89 ± 0.06^{a}	135.49 ± 0.36^{b}	4.16 ± 0.04^{b}	22.33 ± 1.33^{c}	22.49 ± 0.55^{a}	3.28 ± 0.07^{a}	68.45 ± 0.20^{c}	Loam

^{TOC}Total Organic Carbon, ^{WHC}Water Holding Capacity. Values represent means ± SD, significance difference is at $p < 0.05$, different letter assigned on the same column shows significance difference

From the analysis, OGF had the highest nitrogen level of 0.38±0.01% whereas farms 139 and 242 had the lowest N content (0.34±0.03 and 0.34±0.02%, respectively). However, there was no significant difference ($\rho > 0.05$) between content for farms 139, 242, and 13010 (Table 1). Also, farm13010 and 212 had no significant differences ($\rho > 0.05$) in % N. Farm OGF had significance difference nitrogen content compared to the rest of the farms. Farm 13010 had the highest phosphorous level of 144.39±0.32 mg kg^{-1} while farm 15030 had the lowest phosphorous level of 21.48±0.03 mg kg^{-1}. There was no significant difference ($\rho > 0.05$) in means between farms 15030 and 139 (21.48±0.03 and 21.49±0.02 mg kg^{-1}, respectively), however, all other farms differed significantly in phosphorous content. Farm OGF had the highest potassium level of 44.37±0.45 mg kg^{-1} and farm 139 had the lowest level of 1.41±0.01 mg kg^{-1}. There were no significant differences in means of potassium ($\rho > 0.05$) between farms 212 and 139 (1.44±0.09 and 1.41±0.01 mg kg^{-1}, respectively), farms 242 and 13010 (13.79±1.10 and 13.59±0.18 mg kg^{-1}, respectively), however, there were significant differences in potassium level in farm 15030 and OGF with the rest of the farms. The highest calcium level was noted in farm OGF (67.89±0.06 mg kg^{-1}) and the lowest in farm 139 (0.55±0.02 mg kg^{-1}). There were no significant differences ($\rho > 0.05$) in calcium levels in farms 212 and 15030 (6.02±0.01 and 6.48±0.26 mg kg^{-1}, respectively), and farms 139 and 242 (0.55±0.02 and 0.62±0.00 mg kg^{-1}, respectively). However, farm 13010 and OGF (46.43±0.38 and 67.89±0.06 mg kg^{-1}, respectively) were significantly different from other farms. Farm 242 recorded the highest amount of Mg of 216.59±0.28 mg $kg^{-1}$and lowest Mg was recorded in farm 15030 (101.20±0.14 mg kg^{-1}). There was a significant difference ($\rho > 0.05$) in Mg levels in all farms.

All the soils from the six farms were acidic. The highest pH reading was recorded in farm 13010 (5.05±0.10) while the lowest was recorded in farm OGF (4.16±0.04). There was no significant different in pH level between farms 212, 139, 13010 and 15030. Similarly, there were no significant differences in pH level in farms 212, 242, and OGF. However, pH levels of farms OGF and 242 were significantly different from pH levels of farms 139, 13010 and 15030.

4.2 Toxicity effects of hexazinone and 2,4-D on viable bacterial density

Colony forming units (CFUs) were enumerated after treating soil with hexazinone and 2,4-D. Plate 1 shows the observed effects of hexazinone and 2,4-D on CFUs compared to control.

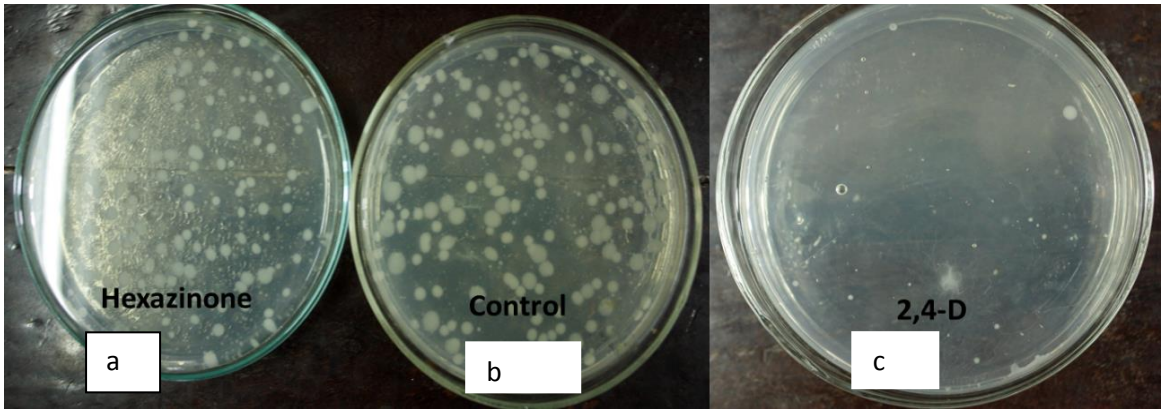

Plate 1: Bacteria CFUs on nutrients agar treated with hexazinone, 2,4-D and a control after the seventh day of incubation

The results showed that there was gradual decrease in bacterial density as the days increased for 2,4-D treated soil samples. For the control soil samples (without treatment with either hexazinone nor 2,4-D) there was no significant difference ($\rho < 0.05$) in CFUs for five subsequent days of incubation ($2.49\pm5.03\times10^5$ g^{-1} soil to $2.41\pm3.18\times10^5$ g^{-1} soil). The suppression of the bacterial growth was observed in both hexazinone and 2,4-D, with significantly higher suppression being noted in 2,4-D treated soils. There was significance ($\rho < 0.05$) decrease in CFUs in day one ($2.53\pm5.13\times10^5$ g^{-1} soil) in hexazinone treated soils. This observed growth suppression effects lasted for a period of three days with a decline in CFUs reading to $1.22\pm2.89\times10^5$ g^{-1} soil. The suppression effect of hexazinone to bacterial growth was overcome on day four ($1.89\pm8.29\times10^5 \pm g^{-1}$ soil), with an observed increased bacterial growth indicated by notably high CFUs by day seven ($3.51\pm6.69\times10^5$ g^{-1} soil), which was a significant ($\rho < 0.05$) compared to the rest of the days. There was suppression of bacteria growth by 2,4-D was as noted in hexazinone. However, the adverse effects of 2,4-D were higher by 10 log units compared to hexazinone with the first day of treatment recording $1.88\pm7.45\times10^5$ CFUs g^{-1} soil. The suppression lasted for three days, with a decline to $1.33\pm1.33\times10^4$ g^{-1} soil. This was preceded with a recovery from suppression with highest recording of CFUs noted in day seven ($2.00\pm5.13\times10^4$ g^{-1} soil). Though, there was recovery of the microorganisms from day four up to day seven, there was no significance difference ($\rho > 0.05$) in the recorded means for this period. Table 2 shows mean variation in colony forming units (CFUs) for soil samples treated with hexazinone and 2,4-D and a control soil sample with no herbicide treatment.

Table 2: Densities of viable bacteria (CFUs g^{-1}) in soil recorded for seven consecutive days following herbicide treatment in an out-grower farm (OGF)

Days	Untreated soil CFUs*10^5 g^{-1} soil	Hexazinone treated soil CFUs*10^5 g^{-1} soil	2,4-D treated soil CFUs*10^5 g^{-1} soil
1	2.49 ± 5.03^{abc}	2.53 ± 5.13^{c}	1.88 ± 7.45^{a}
2	2.54 ± 4.67^{ab}	2.09 ± 3.84^{d}	$3.50\pm8.15*10\text{^}4^{b}$
3	2.56 ± 0.88^{ab}	1.67 ± 51.97^{e}	$1.97\pm2.96*10\text{^}4^{bc}$
4	2.63 ± 6.64^{a}	1.89 ± 8.29^{d}	$1.33\pm1.33*10\text{^}4^{bc}$
5	2.41 ± 3.18^{abc}	2.61 ± 6.00^{c}	$1.67\pm2.03*10\text{^}4^{bc}$
6	2.28 ± 2.72^{c}	3.11 ± 4.10^{b}	$1.70\pm2.31*10\text{^}4^{bc}$
7	2.33 ± 2.73^{bc}	3.51 ± 6.69^{a}	$2.00\pm5.13*10\text{^}4^{bc}$

Values are means ±SD, n = 3, Means followed by the same letter in the same column are not significantly different at 5% LSD

4.3 DHA activity

To quantify the amount of TPF present in the samples, a standard calibration curve was prepared using standard formazan (Figure 7) with correlation coefficient R^2 of 0.9997.

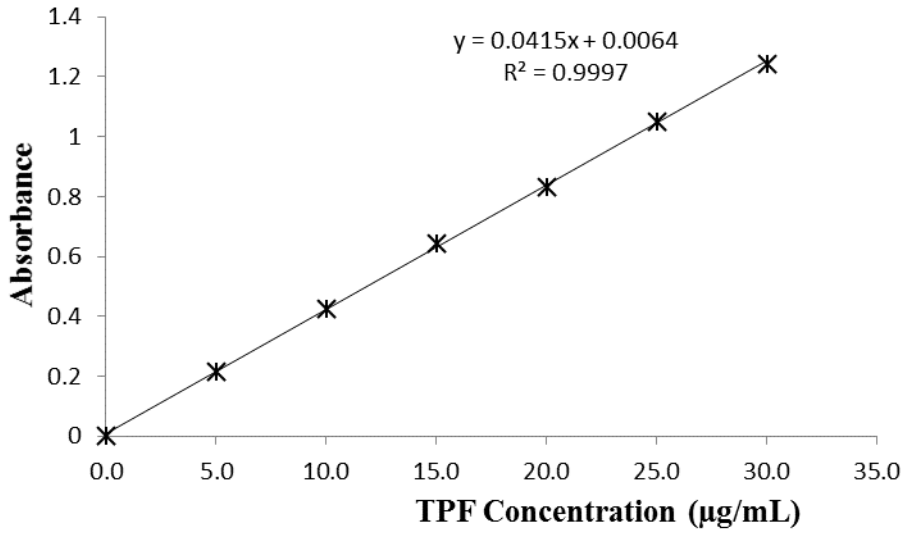

Figure 7. Standard calibration curve for TPF

The overall analysis of the TPF concentration showed that hexazinone treated soils had higher DHA activity across all farms compared to the other two treatments. Soil treated with 2,4-D recorded the least DHA activity in all farms. Table 3 shows the overall mean concentrations and the standard deviations for the TPF concentrations across the three farms 139, 212 and OGF without treatment with hexazinone and 2,4-D and after the treatment.

Table 3: The overall mean concentrations and std.dev of TPF in µg g^{-1} Soil soils from the farms for the seven days treatments period

Treatment	Replicates	139	212	OGF
2,4-d	21	0.53^{c}±0.12	0.54^{c}±0.14	6.59^{b}±1.18
Hexazinone	21	16.38^{a}±1.82	21.97^{a}±3.45	113.45^{a}±15.45
Untreated	21	4.53^{b}±0.41	6.10^{b}±0.34	21.58^{b}±3.23

Means followed by the same letter in the same column are not significantly different at 5% LSD

In farm 139 there was a significant difference in enzyme activity for the three treatments. Hexazinone treated soils performed best with 57% higher DHA activity as compared to the control which had 4.53±0.41µg TPF g^{-1} soil. Plate 2 shows methanolic extracts of TPF from the treated and untreated soil samples. On the contrary, the soil sample treated with 2,4-D in farm 139 had higher inhibition of DHA in reference to the control samples, recording 78.98% decrease in DHA activity. In farm 212, there was also significant difference in DHA activity within all the treatments with soil sample treated with hexazinone having higher activity of DHA by 56.52% (6.10±0.34 µg g^{-1} soil) with reference to the untreated soil. On the other hand, 2,4-D had suppressing effects on DHA activity by 83.71% with reference to the untreated soil sample. For the OGF, which had no history of herbicide treatment, DHA was significantly different for all the treatments. Hexazinone recorded the highest positive activity by 67.53% with reference to untreated soil sample, while 2,4-D had negative effects on DHA activity by 53.37% with reference to the untreated soil sample. From the experiment, there was clear evidence, of the negative effects on enzyme activity in all farms upon application of 2,4-D, while hexazinone had boosting effects on the enzyme activity in all farms.

(a)

(b)

Plate 2: (a) Clear extracts from soil samples treated with 2,4-D herbicides, indicating little formation of formazan (b) Deep pink colour formation of formazan from extracts of soil samples treated with hexazinone.

Figure 8 shows comparison of mean concentrations of TPF within the incubation period of seven days for the three farms 139, 212 and OGF without treatment with hexazinone or 2,4-D and upon treatment with the two herbicides. TPF concentration for farm 212 without treatment varied from 3.20±0.13 µg g^{-1} soil in day one to 8.09±0.18 µg g^{-1} soil in day seven. In farm 139 the concentration of TPF without treatment varied from 1.06±0.13 µg g^{-1} soil in day one to 6.88±0.18 µg g^{-1} soil in day seven. In farm OGF the TPF concentration varied from 5.81±0.30 µg g^{-1} soil in day one to 41.29±0.12 µg g^{-1} soil in day seven. There was significant difference ($\rho < 0.05$) in DHA between farm OGF and 212, and also between OGF and 139 for untreated soils samples as indicated by significant differences in the means of TPF concentrations (Appendix 1). However, there was no significance difference at $\rho < 0.05$ in DHA activity between farm 212 and 139.

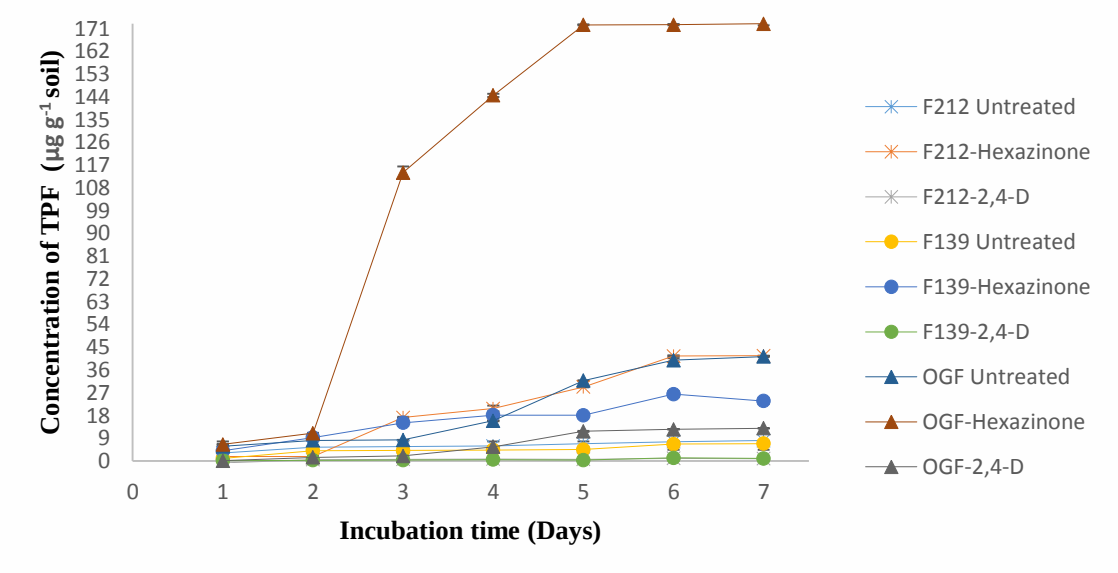

Figure 8. TPF concentration in soils with and without the addition of herbicides in farms OGF, 212 and 139. Data points represent means for three replicates.

The TPF concentration in 212 treated with hexazinone ranged from 1.79±0.13 µg g^{-1} soil in day one to 41.62±0.0.07 µg g^{-1} soil in day seven. In farm 139, the concentration upon treatment with hexazinone ranged from 4.0±0.37 µg g^{-1} soil in day one to 23.68±3.18 in day seven and that of farm OGF ranged from 6.55±0.12 µg g^{-1} soil in day one to 172.95±0.60 µg g^{-1} soil in day seven. There was significant difference ($\rho < 0.05$) in DHA activity from day 3

between OGF–hexazinone treated soil and 212 and 139-hexazinone treated soil. There were no significant changes ($\rho < 0.05$) in DHA activity between 212-hexazinone and 139-hexazinone treated soils. On the other hand, there was a remarkable and significant increase ($\rho < 0.05$) in DHA activity upon treatment of soil sample with hexazinone for all the three farms.

Treating soils with 2,4-D had an effect on DHA activity. The concentration of TPF in 212 upon 2,4-D treatment ranged from 0.82±0.13 µg g^{-1} soil in day one to 0.92±0.23 µg g^{-1} soil in day seven. Means concentration of farm 139 upon treatment with 2,4-D ranged from 0.12±0.12 µg g^{-1} soil in day one to 0.99±0.57 µg g^{-1} soil in day seven and that of farm OGF ranged from 0.05±0.18 µg g^{-1} soil in day one to 12.91±0.07 µg g^{-1} soil in day seven. The DHA activity in OGF (6.59±1.18 µg g^{-1} soil) upon treatment with 2,4-D was significantly higher ($\rho < 0.05$) as compared to the other two farms. There was no significant difference in DHA activity between 212 and 139 (0.54±0.14 and 0.53±0.12 µg g^{-1} soil) at $\rho < 0.05$ level. These DHA activities upon treatment with 2,4-D were significantly lower ($\rho < 0.05$) in all the farms 212, 139 and OGF compared to when the soil was not treated with any of the herbicides and when the soils were treated with hexazinone.

A Pearson's correlation was done to determine the relationship between the bacterial density and the DHA activity (Table 4). There was positive correlation between CFUs and DHA activity upon application of hexazinone in two farms (r=0.67, p=0.0008 in farm OGF, r=0.54, p=0.0109 in farm 212), however, there was no correlation in CFU and DHA upon application of herbicide in farm 139. On the other hand, there was negative correlation between CFUs and application of 2,4-D in all farms. The implication of these correlation data is that, the application of hexazinone herbicide boosted the activity of the DHA enzymes. However, the application of 2,4-D herbicide had diminishing effects on the DHA activity as shown by the negative correlation.

Table 4: Correlation matrix between colony forming units (CFUs) and DHA activity

1		H-CFU	D-CFU	U-CFU	OGF-U-DHA	OGF-H-DHA	OGF-D-DHA	212-U-DHA	212-H-DHA	212-D-DHA	139-U-DHA	139-H-DHA	139-D-DHA
CFU-Hex	PC-r	1.00000	0.148	-0.290	0.674***	0.309	0.598**	0.397	0.544*	0.304	0.402	0.334	0.340
	Sig. (2tailed)		0.5215	0.2018	0.0008	0.1729	0.0042	0.0744	0.0109	0.1801	0.0708	0.1392	0.1314
CFU-2, 4-D	PC-r		1.00000	0.284	-0.478*	-0.696***	-0.539*	-0.780***	-0.586**	0.005	-0.769***	-0.640***	-0.377
	Sig. (2tailed)			0.2130	0.0285	0.0005	0.0116	<.0001	0.0053	0.9844	<.0001	0.0018	0.0918
CFU-Untreated	PC-r			1.00000	-0.461*	-0.332	-0.459*	-0.407	-0.404	-0.304	-0.364	-0.487*	-0.223
	Sig. (2tailed)				0.0354	0.1409	0.0363	0.0671	0.0694	0.1810	0.1051	0.0253	0.3306

[H] Hexazinone, [D]2,4-D, [U] Untreated, [PC-r] Pearson Correlation and [Sig] Significance, *Correlation is significant at 0.05 level (2-tailed) **Correlation is significant at 0.01 level (2-tailed) ***Correlation is significant at 0.001 level (2-tailed), [212]212, [139]139 and [CFU]Colony Forming Unit.

4.4 Herbicide residues in soil

Calibration curves for hexazinone, 2,4-D and 3,5-DCC were obtained after running HPLC of serial standard dilutions prepared from the stock solutions (Figures 9-11). The calibration curves had high correlation coefficient ($R^2 > 0.986$) implying a good linearity for use in sample quantification. The retention time for the three compounds were 5.2, 7.5, and 8.2 minutes for hexazinone, 2,4-D and 3,5-DCC, respectively.

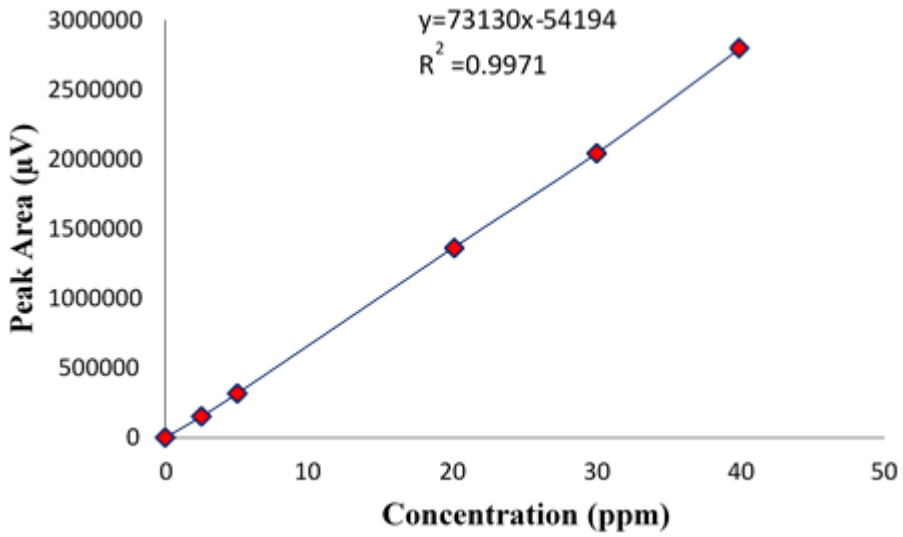

Figure 9. Standard calibration curve for hexazinone

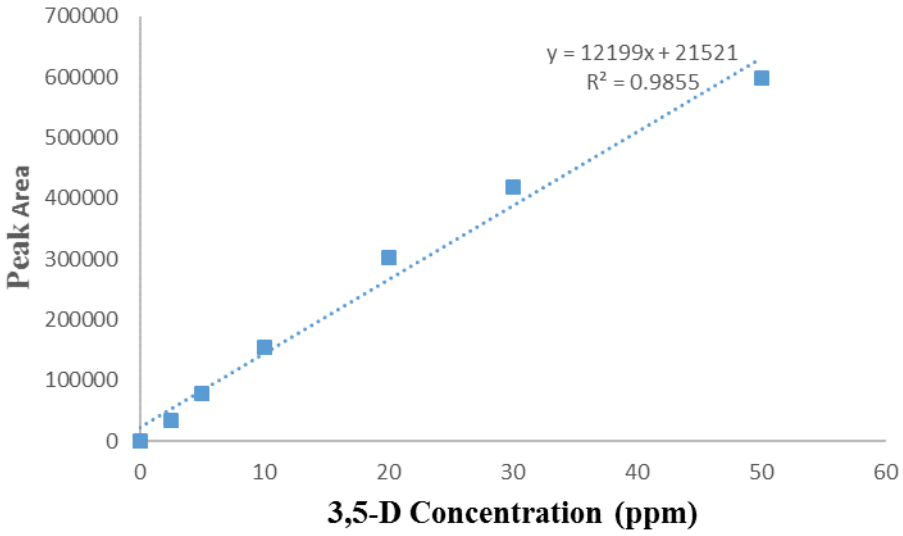

Figure 10. Standard calibration curve for 2,4-D

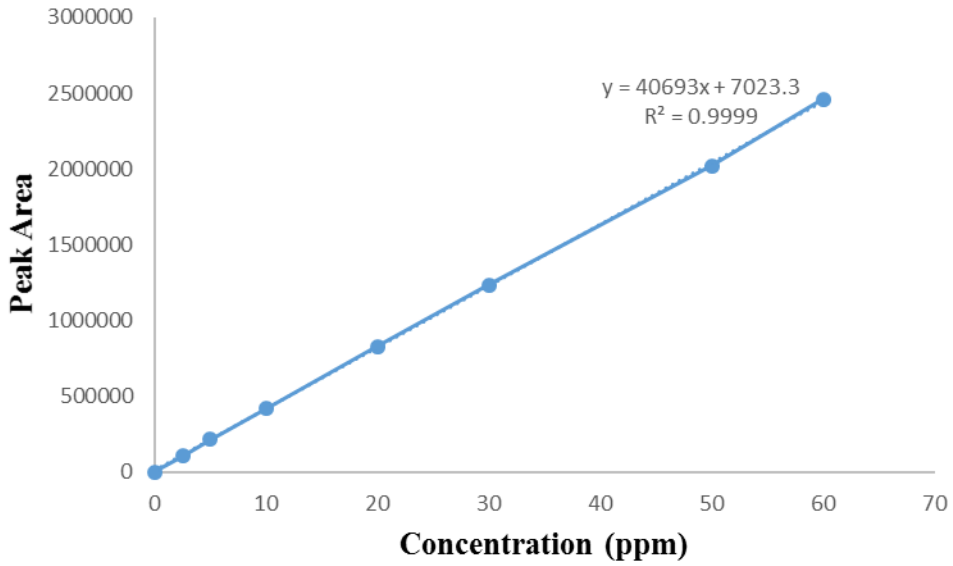

Figure 11. Standard calibration curve for 3,5-DCC

Soxhlet extraction of herbicides residues from the soil showed the presence of all the herbicides in question, with an additional of one of 2,4-D metabolite 3,5-DCC as shown in the table 5. There were high residues of hexazinone obtained from farm 212 (10.43±0.24 µg g^{-1} soil). Farm 139 had high 2,4-D and its metabolite residues compared to farm 212.

Table 5: Herbicides residues from sampled soil

	Herbicides Residues		
Farms	**Hexazinone (µg g^{-1} soil)**	**2,4-D (µg g^{-1} soil)**	**3,5-DCC (µg g^{-1} soil)**
Farm 212	10.43±0.24	6.25±0.03	4.29±0.02
Farm 139	5.45±0.03	7.19±0.04	5.48±0.09

4.5 Bacterial degradation of herbicides

4.5.1 Isolates from various farms

A total of seven bacteria isolates were obtained from the six selected farms using mineral salt medium amended with hexazinone as the sole source of carbon and nitrogen and 2,4-D as the sole source of carbon. From the selective liquid enrichment media with MSM supplemented with 50 mgL^{-1} hexazinone, four distinct bacterial strains were isolated. Isolates N212H4, N15030H3 and N15030H2were isolated from farms 212 and 15030 whereas, N13010H1 was isolated from soils obtained from farm 13010. In order to isolate 2,4-D

tolerant bacteria MSM was amended with 60 mgL^{-1} of this compound. Three bacteria isolates coded as N13010D3, N139D1 and N13010D4 tolerant to 2,4-D were obtained from farm 139 for N139D1and from farm 13010 for the other isolates, respectively.

4.5.2 Growth and hexazinone degradation by the four strains

The growth and the ability of the four bacteria strains to degrade hexazinone were evaluated through liquid culture incubation experiments for 28 days. The degradation ability of hexazinone was observed in four isolates encoded as N13010H1, N15030H2, N15030H3 and N212H4 (Figure 12 a-d). N13010H1 was able to degrade hexazinone in liquid culture up to 64.76 % of the initial concentration. The degradation of hexazinone was accompanied by growth of the N13010H1 isolate as indicated by increase in the optical density (OD_{600} nm) from 0.27 to 0.70. The same trend was also observed in the other three bacteria isolates with N15030H2 degrading hexazinone by 57.58% of the initial concentration and an OD_{600} increase of cells from 0.25 to 0.55. Isolate N15030H3 and N212H4 were the best single isolates degraders for the chemical with N15030H3 recording a degradation percentage of 82.00% of the initial concentration and an OD_{600} nm from 0.25 to 0.83 by the 28^{th} day of incubation. The N212H4 was able to degrade hexazinone by 79.84% of the initial concentration with a concomitant increase in OD_{600} nm from 0.25 to 0.60.

The rate of degradation as well as the half-life of each bacteria strain was determined by first order rate kinetics. The analysis of degradation rate by the four isolates showed that there was no significance difference ($p < 0.05$) in degradation rates of N15030H3 and N212H4 isolates, which was 0.068 mg mL^{-1} day^{-1} at a half-life of 10.2 days and 0.06 mg mL^{-1} day^{-1} at a half-life of 10.8 days, respectively (Table 6). There was a significance difference in degradation rates between N13010H1 and N15030H2 with 0.0417 mg mL^{-1} day^{-1} and a half-life of 16.62 days for the former and 0.04 mg mL^{-1} day^{-1} and a half-life of 18.24 days for the latter, respectively. Similarly, there was observed significant difference ($P < 0.05$) in the rates of degradation as well as half-life between N13010H1 and N15030H2, N15030H3 and N212H4 and also between N15030H2 and N13010H1, N15030H3 and N212H4.

Table 6. First order kinetics rates for the four bacteria isolates and their consortia

Isolate	**Rate constant (k) mg mL^{-1} day**	**Half-life (days)**	**Correlation Coefficient (R^2)**
N13010H1	0.0417	16.62	0.9782
N15030H2	0.0380	18.24	0.9311
N15030H3	0.0681	10.18	0.9685
N212H4	0.0641	10.81	0.9583
Mixed Bacteria Culture (MBC)	0.1520	4.57	0.8818

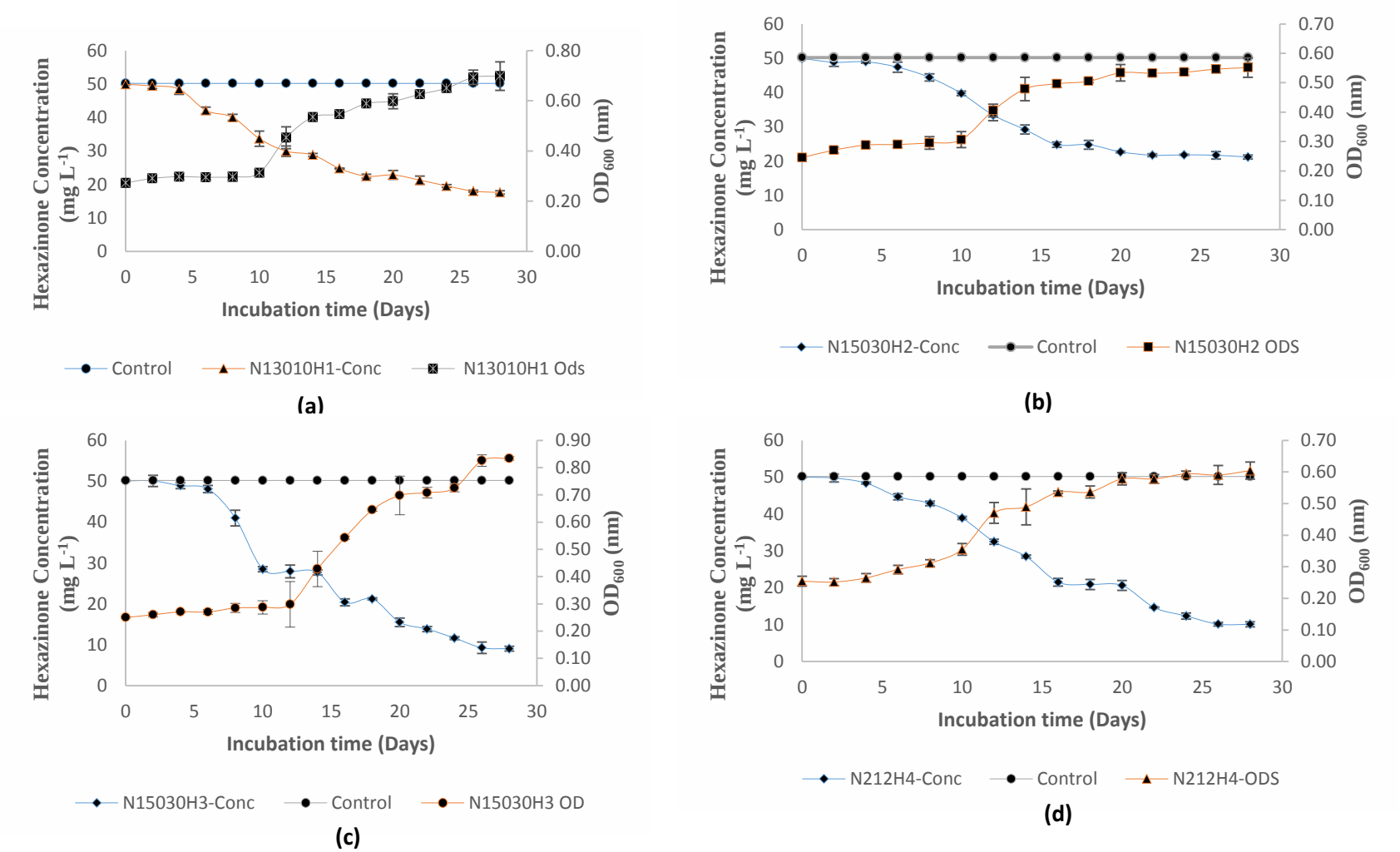

Figure 12. Growth and degradation curves of hexazinone by pure isolates; a-N13010H1, b-N15030H2, c-N15030H3 and d-N212H4 in liquid cultures

4.5.2.1 Degradation of hexazinone by mixed bacterial culture (MBC)

Considering the natural environment, these pure bacteria isolates do not exist as single microbes but there must be cohabitation with other bacteria, and therefore this necessitated the study for the synergistic degradation among the four strains. The consortium of the four bacteria strains was therefore prepared and the experiment carried under the same condition as those of the single strains. The utilization of hexazinone was determined by examining the growth using the optical density (OD_{600}) measurements and the quantification of residual hexazinone by HPLC. The growth of the consortium and the decrease in residual hexazinone in MSM is shown in Figure 13. The results demonstrated that the growth of MBC (N13010H1, N15030H2, N15030H3 and N212H4) was accompanied by a steady disappearance of the hexazinone from the MSM. The OD_{600} increased from 0.28 in day one to 1.53 in day 28. This showed there was synergism of MBC in utilization of the hexazinone as the sole source of carbon and nitrogen, than using a single strain of bacteria. The degraded amount was 98.96% of the initial concentration, which shows almost complete degradation of hexazinone by MBC by the time of termination of the experiment. The MBC also recorded higher degradation rates of 0.152 mg mL^{-1}/day^{-1} at a half-life of 4.57 days.

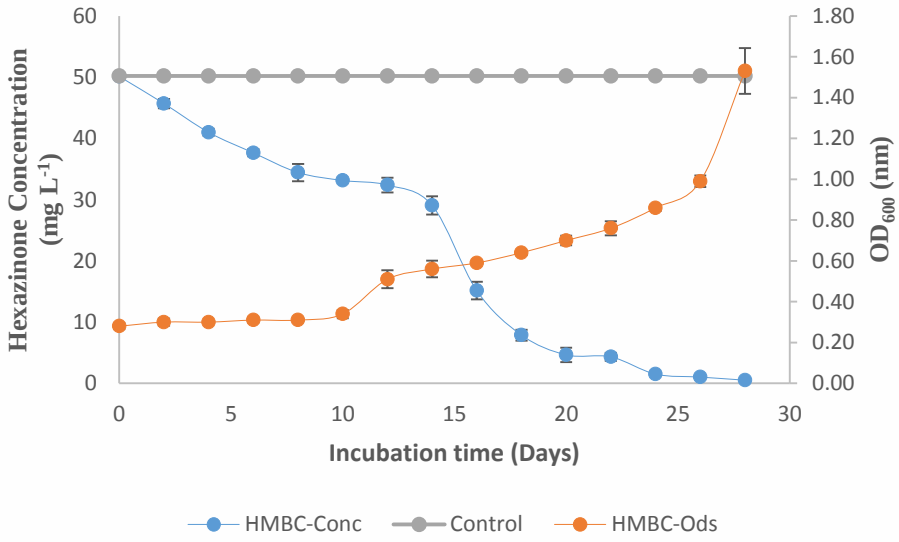

Figure 13. Growth and degradation curves by hexazinone degrading mixed bacterial culture (HMBC) in liquid cultures

4.5.3 Growth and degradation of 2,4-D by single bacterial isolate and mixed bacterial culture (MBC)

Four bacteria were isolated with three of them showing high capacity to biodegrade 2,4-D in MSM. Isolate N13010D3 showed the best degradation capacity with 82.3% reduction of the parent 2,4-D compound by 28th day. The growth of N13010D3 was followed

by monitoring the changes in OD_{600} which increased from 0.08 to 0.22 nm (Figure 14). The increase in bacterial cells of N13010D3 isolate was characterized by a lag phase of 7 days. N13010D4 was the second-best isolate degrading 2,4-D compound by 74.3% of the initial concentration in the MSM by the time the experiment was terminated. Increase in optical density from 0.10 to 0.18 after 28 days of incubation was indicative of utilization of 2,4-D as sole source of carbon by the isolate for its cell multiplication. There was no any noted lag phase in cell growth of N13010D4 isolate in MSM medium. N139D1 showed the lowest degradation capacity of 65.2% reduction in the 2,4-D concentration by the end of the incubation period for the experiment. N139D1 also was characterized by increase in cell density at OD_{600} nm from 0.08 to 0.15 nm by the end of the experiment. The consortium of the bacterial degraders in MSM media amended with 60 mgL^{-1}2,4-D demonstrated reduction of 2,4-D by 84.54% of the initial concentration. The degradation was accompanied by an increase in growth of the bacterial consortium, indicated by a change in OD_{600} nm from 0.10 to 0.21 nm and release of different metabolites (Figure 15). The percentage decline of 2,4-D in MSM as a result of degradation by the bacterial consortium was significantly different from the degradation observed in the three single isolates. The degradation of 2,4-D by the N139D1, N13010D3, N13010D3 and their consortium showed presence of 3,5-DCC.

The rate of degradation as well as the half-life of each bacteria strain was determined by first order rate kinetics (Table 7). There was significance difference ($\rho < 0.05$) in degradation rate between N139D1, N13010D3 and N13010D4. N13010D3 recorded the highest degradation potential of 0.04µg mL^{-1} day^{-1} at a half-life of 15.95 days (table 7). N13010D4 degraded 2,4-D at a rate of 0.04 µg mL^{-1} day^{-1} at a half-life of 17.91 days. N139D1 had the longest half-life of 19.53 days at a degradation rate of 0.04 µg mL^{-1} day^{-1}. The bacterial consortium recorded a degradation rate of 0.06µg mL^{-1} day^{-1} at a half-life of 12.14 days. The rate of degradation of 2,4-D by MBC was significantly different compared to those of the three-single strain.

Table 7. First order kinetic rates and half-life for the four bacteria isolates and their consortium

Isolate	**Rate constant (k) µg mL^{-1} day^{-1}**	**Half-life (days)**	**Correlation Coefficient (R^2)**
N139D1	0.04[d]	19.53[a]	0.96
N13010D3	0.04[b]	15.95[c]	0.79
N13010D4	0.04[a]	17.91[b]	0.85
MBCD	0.06[c]	12.14[d]	0.97

Means followed by the same letter in the same column are not significantly different at 5% LSD

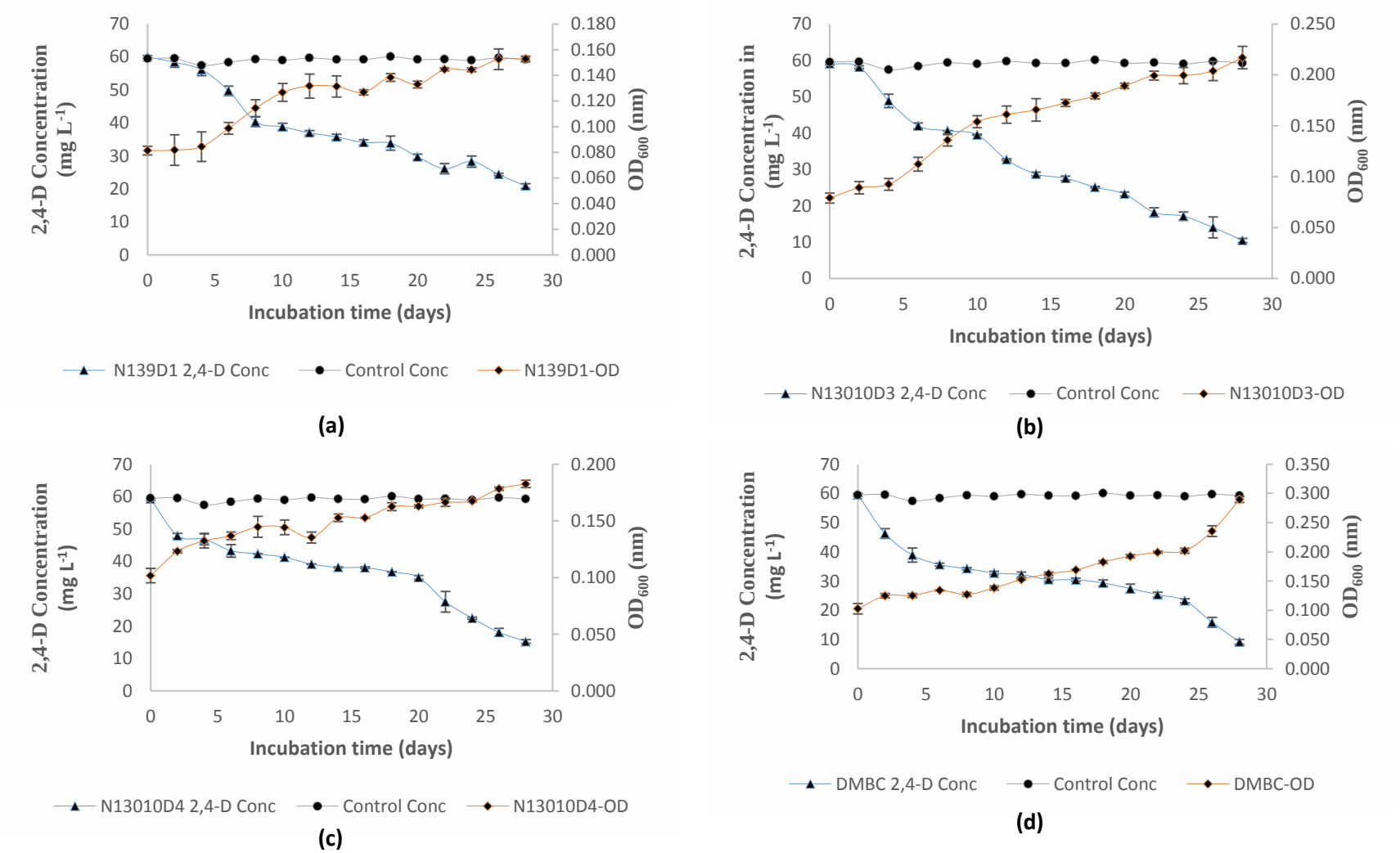

Figure 14. Growth and degradation curves of 2,4-D by pure bacteria isolates a-N139D1, b-N13010D3, c-N13010D4 and a consortium of 2,4-D degrading bacteria (D-MBC) in liquid cultures

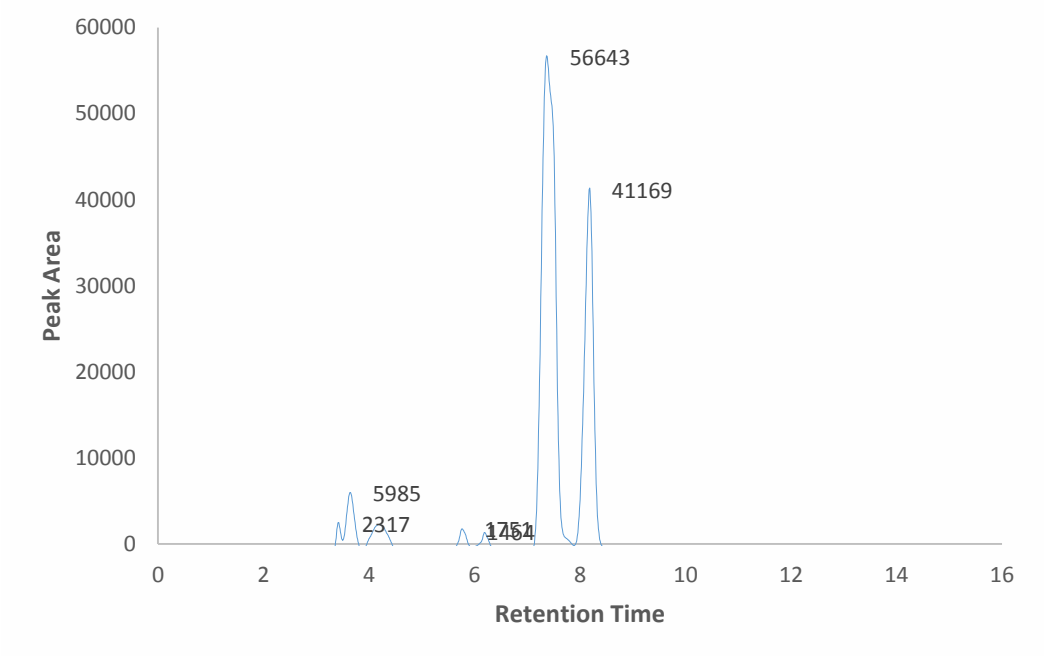

Figure 15. ChromatoGram of MBC liquid cultures for 2,4-D by the 28th Day of incubation

4.6 Morphological and biochemical characterization

4.6.1 Morphological and biochemical characteristics of hexazinone bacterial isolates

Morphological characterization was based on Gram staining which revealed that N13010H1 was Gram-positive rods shaped. The Biochemical tests showed the isolate was catalase negative, gelatinase negative, with no ability to hydrolyze starch and indole positive. N15030H2 was Gram-negative and rod-shaped bacteria. Biochemical test showed the organisms was catalase positive and negative on gelatin, starch and indole hydrolysis (Plate 3 and 4). N15030H3 was Gram-positive bacilli bacteria. Biochemical test showed that it was catalase positive and able to hydrolyze gelatin, and starch. N15030H3 gave negative result on the ability to hydrolyze indole. The final isolate was N212H4 which had cocci morphology and Gram-positive. Biochemical test on N212H4 showed that the isolate was catalase positive. N212H4 gave negative test on indole hydrolysis. However, the isolate was able to hydrolyze gelatin and starch. For the validation of the tests, morphological as well as the biochemical tests were run concurrently with two known bacterial reference cultures, *E. coli* (ATCC 25922) and *B. spizizenii* (ATCC 6633) DLD Scientific (Durban North, SA). *E. coli* in this case was Gram-negative, rod shaped bacteria, catalase positive and unable to hydrolyze gelatin and starch but with ability to hydrolyze indole. *B. spizizenii* on the other hand, was Gram-positive and rod shaped. Biochemical test showed *B. spizizenii* was catalase positive,

able to hydrolyze gelatin, starch but negative results on the indole hydrolysis. Morphological and biochemical tests results are shown in table 8.

Table 8. Morphological and biochemical tests for the four bacteria isolates and two known reference bacteria

Biochemical Test	**Bacteria Isolates**					
	N13010H1	**N15030H2**	**N15030H3**	**N212H4**	***E. coli***	***B. spizizenii***
Gram Staining	G+ve Rods	G-ve Rods	G+ve Rods	G+ve Cocci	G-ve Rods	G+ve Rods
Catalase	-ve	+ve	+ve	+ve	+ve	+ve
Gelatinase	-ve	-ve	+ve	-ve	-ve	+ve
Starch Hydrolysis	-ve	-ve	+ve	+ve	-ve	+ve
Indole Test	+ve	-ve	-ve	-ve	+ve	-ve

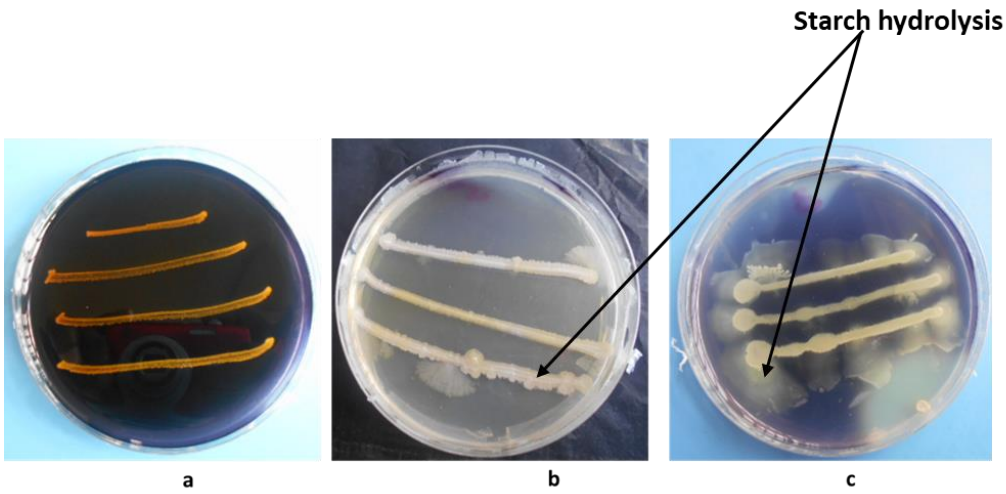

Plate 3: Starch hydrolysis test for a-*E. coli* (negative), b-N15030H3 (positive), and c-N13010H1 (positive).

a b c

Plate 4: Gelatin hydrolysis tests for a-*B. spizizenii*, b-N15030H2, and c-N212H4

4.6.2 Morphological and biochemical characteristics of 2,4-D degrading bacterial isolates

Morphological characterization of N139D1 showed that it was Gram-negative and rod-shaped bacteria. The Biochemical tests showed the isolate was catalase negative with ability to hydrolyze starch and gelatin but gave negative test result on indole hydrolysis (Table 9). N13010D3 was Gram-negative and rod-shaped bacteria. The isolate was catalase positive. N13010D3 gave negative results on gelatin hydrolysis, and indole hydrolysis but was able to hydrolyze starch. N13010D4 was Gram-negative staphylococci bacteria. Biochemical tests showed that it was catalase positive. The isolate gave negative results on the hydrolysis test for gelatin, indole and starch.

Table 9. Morphological and biochemical tests for N139D1, N13010D3 and N13010D4 and two known bacteria reference cultures

Biochemical Test	**N139D1**	**N13010D3**	**N13010D4**	***E. coli***	***B. spizizenii***
Gram Staining	G-ve Rods	G-ve Rods	G-ve Cocci	G-ve Rods	G+ve Rods
Catalase	-ve	+ve	+ve	+ve	+ve
Gelatinase	+ve	+ve	-ve	-ve	+ve
Starch Hydrolysis	+ve	-ve	-ve	-ve	+ve
Indole Test	-ve	-ve	-ve	+ve	-ve

4.7 Molecular characteristics

4.7.1 Phylogenetic analysis of hexazinone degrading bacterial isolates

Before doing the sequencing, the 16S rRNA DNA for the seven isolates and the two reference bacteria (*E. coli* and *B. spizizenii*) were run on agarose gel. Figure 16 shows the results of gel electrophoresis obtained for the samples.

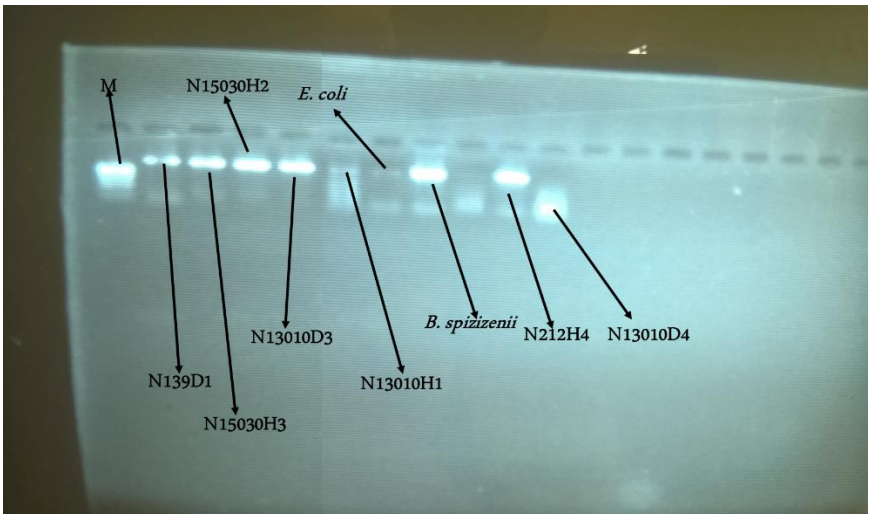

Figure 16. Ethidium bromide-stained agarose gel (1.0%) containing PCR products by Bac8f and Bac1492r universal primers and template DNA from the indicated bacteria strains. Lanes M is 1.0 Kb plus ladder (Invitrogen Corp.).

Molecular Phylogenetic analysis of 16S rRNA genes for hexazinone bacterial degraders revealed five major clusters. Isolates revealed that isolate N13010H1 clustered with *Enterococcus* sp and *Bacterium NLAE zl-H322* with a boostrap range of 77% to 100% (Figure 17). N13010H1 showed a gene similarity of 99% with those deposited in the GeneBank of both strains. The identification was further based on the biochemical test which showed the isolate coincided with those of *Bacterium NLAE-zl-H322* (Chikere and Ekwuabu, 2014). Phylogenetic analysis of N15030H2 showed that the isolate clustered with *Enterobacter* sp such as *Enterobacter cloacae, Enterobacter xiangfangensis,*and *Enterobacter ludwigii* strains with a bootstraps ranging from 82-99% (Figure 17). The isolate had 16S rRNA gene similarity of 97% on the deposited GenBanks in NCBI database with all *Enterobacter* sp strains macthes. On the other hand, the elimanation was based on the biochemical tests done, which all concided with those of *Enterobacter cloacae* reported by Ngigi *et al.* (2014). Molecular characterization of isolate N15030H3 showed that it had high similarity of its 16S rRNA gene of 98% with *Bacillus cereus, Lysinibacillus* sp, *Planococcaceae bacterium,* and *Bacillus* sp strains. The indentity was also supported with boostrap ranging from 67 to 89% (Figure 17). Further biochemical characterization showed

that the isolate was similar to *Bacillus cereus* strain (Souza and Abrantes, 2011). The molecular identification of N212H4 isolate confirmed the isolate was of *Staphylococcus* sp strain. The isolate had a 16S rRNA gene similarity 89%. Phylogenetic analysis had a high boostrap similarity support between the two strains as well, ranging from 89 to 100% (figure 17). The biochemical test showed that the isolate concide with *Staphylococcus aureus* strain characteristics.

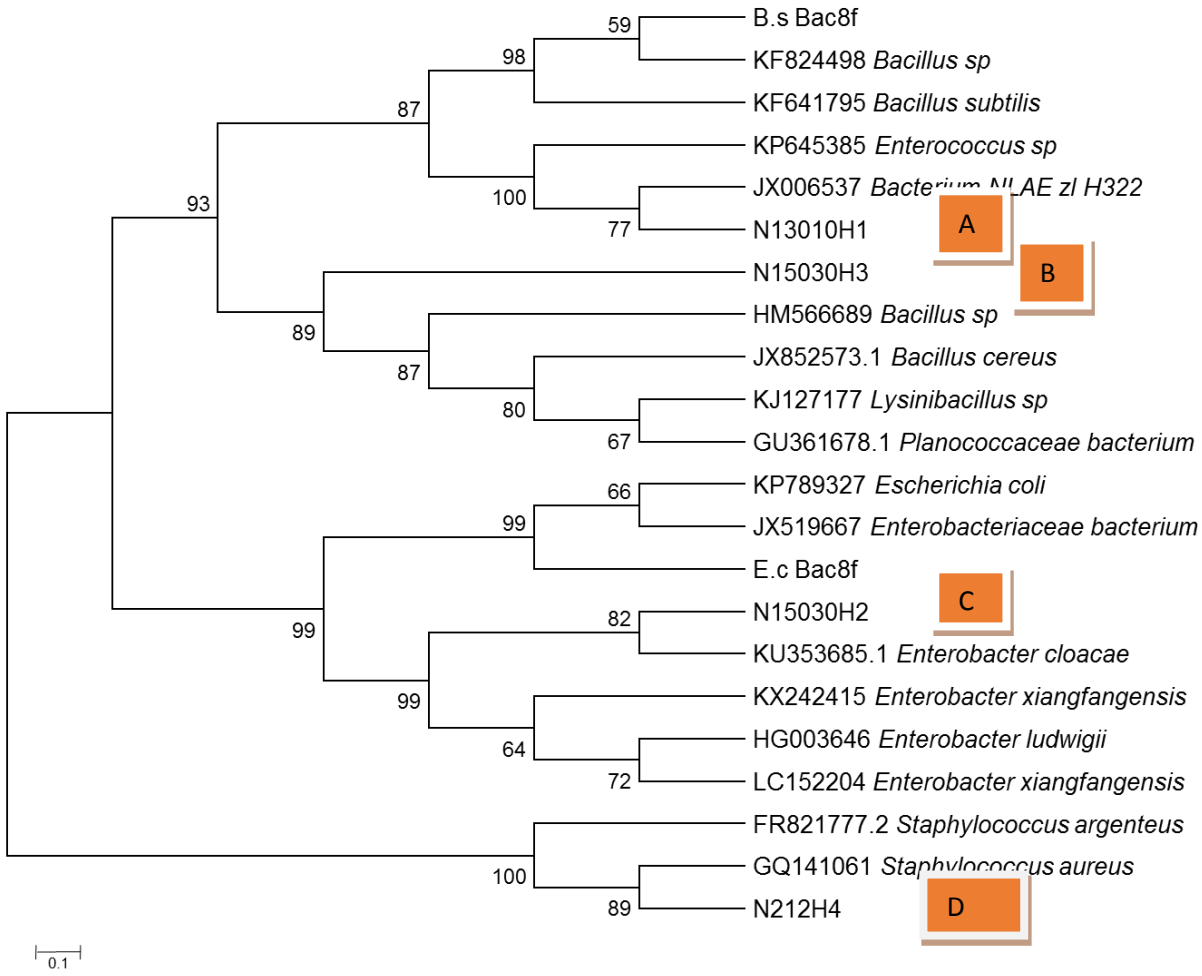

Figure 17. Molecular Phylogenetic analysis for hexazinone bacterial degraders by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a

matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 358 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

4.7.2 Phylogenetic analysis of 2,4-D degrading bacterial isolates

16S rRNA genes analysis of the isolates showed that isolate N139D1 had a high gene similarity with *Bacillus* sp strain accession FJ957634 on NCBI Gen Bank with a similarity score of 97%, Uncultured Bacterium accession KF110301 with a similarity score of 98% and *Bacterium NLAE-zl-P653* with an accession number JQ607502 with a similarity score of 98%. Phylogenetic analysis of N139D1 16S rRNA gene with those of 2,4-D biodegraders and their NCBI GenBank entities showed the isolate clustered with the three mentioned entities with a bootstrap ranging from 58 to 99% (Figure 18).

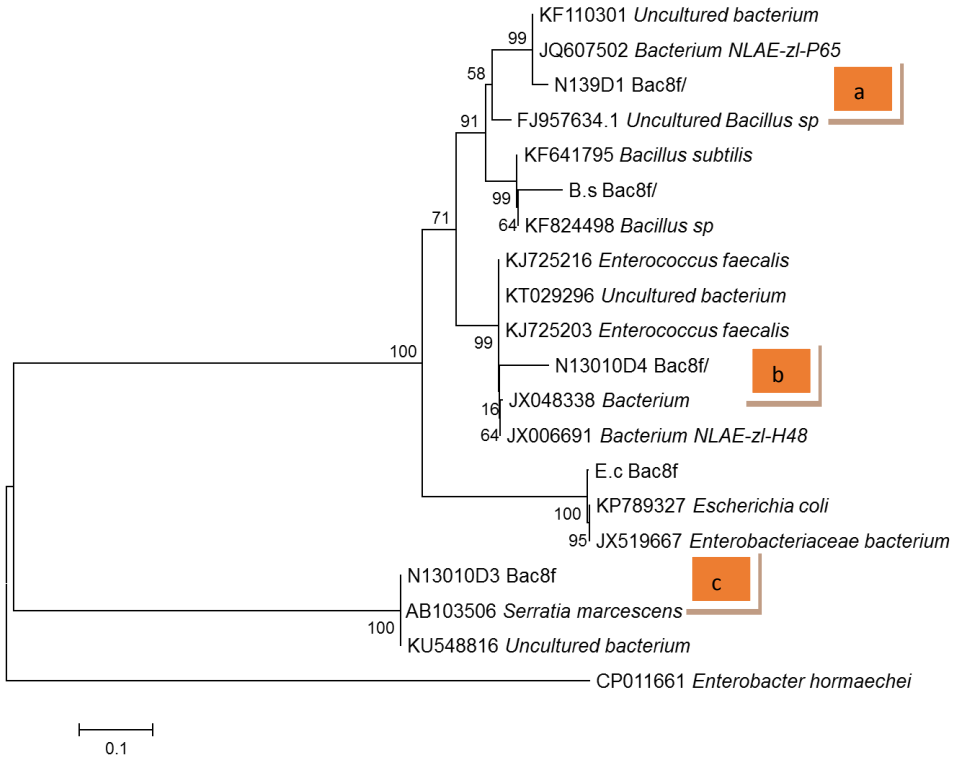

Figure 18. Molecular Phylogenetic analysis by Maximum Likelihood method for 2,4-D bacterial degraders and their similar entities.

The phylogenetic analysis of 2,4-D bacterial degraders revealed six major clusters. 16S rRNA gene in the NCBI GenBank of bacterial isolate N13010D3 showed that the organism had a high gene similarity of 99% with *Serratia marcescens* strain accession

number AB103506, *Enterobacter hormaechei* strain, accession number CP011661 and Uncultured bacterium accession number KU548816. Phylogenetic tree analysis showed that isolate N13010D3 clustered with *Serratia marcescens* and Uncultured bacterium with a bootstraps range of 31 to 100% (Figure 18). On the other hand, the inferred evolutionary history on the phylogenetic tree showed there was distant ancestry between N13010D3 and *Enterobacter hormaechei* as they only shared clade origin. To confirm the identity of N13010D3, both morphological, biochemical and molecular tests were used which showed the bacteria isolate was similar to those of *Serratia marcescens* strain (Whitman *et al.*, 2012*).* The isolate N13010D4 had a 16S rRNA gene similarity of 96-97% with *Enterobacter faecalis* strain, Uncultured bacteria clone and *Bacterium NLEA-zl-G340.* Phylogenetic analysis showed the isolate clustered with the three 16S rRNA genes with a bootstrap value range of 16 to 99% (Figure 18). Biochemical characteristics showed that though N13010D4 isolate gene had similarity with these three organisms, none had similar biochemical and morphological features as such and therefore the organism stand as Uncultured bacteria clone.

CHAPTER FIVE
DISCUSSION

5.1 Soil physicochemical parameters

Soil physicochemical parameters are important factors in biodegradation experiments since they can be used as inferences for the outcomes of such experiments. As the mineralization of the pesticides depends on microorganisms, their activity largely depends on these environmental factors (Shahgholi, and Ahangar, 2014). These soil properties can also be used to explain some soil anomalies that may be observed during analysis. The soil pH ranged from 4.16 to 5.05 indicating all the farms had acidic soil. Biodegradation of some of the pesticides have been found to be slow at pH above 6 and optimum at pH below 5 (Schoenholtz *et al*., 2000). However, the pH effect is relative to the individual compound being degraded and the potential organism which degrade it. The soil phosphorous for five farms were within the medium range (20-40 mg kg^{-1}) with only one field with excess of 144 mg kg^{-1} (>100) (Horneck *et al*., 2011). The nitrogen content was also very low (<5%) (Galloway, 2010). Similarly, the K content was extremely low for all the farms (<150 mg kg^{-1}) and magnesium was within a medium range of 60–300 mg kg^{-1} (Horneck *et al*., 2011). Low level of phosphorous, potassium, calcium and nitrogen may be attributed to acidity of soil (Locascio, 2000; Schoenholtz *et al*., 2000) as was the case in this experiment. Other factors that may lead to low nutrient content in soil are vegetation cover, agricultural activities such as application of fertilizer and clearing of vegetation by burning which are common practices in Nzoia sugar company nuclear estates (Ezeigbo *et al*., 2013). Total nitrogen may also be low in acidic soils since in acid condition there is unavailability of NH_4^+ (Medinski, 2007). The soil temperature ranged between 21-27 ^oC. The major factor that affect the soil temperature is the weather condition such as sun heating which also affect the moisture content (the moisture content ranged between 16 and 22%) as well (Ezeigbo *et al*., 2013). The temperatures of soil play a major role in the degradation of the pesticides. It has been reported that most of the degradation of pesticides tends to increase with increase in temperature between 10 to 45 ^oC (Rani and Sud, 2015). Soil physicochemical properties such as temperature, humidity, and moisture content affect the rate of decomposition of herbicides in soil (Milosevia and Govedarica, 2000). According to Shahgholi and Ahangar (2014), soil moisture content is very crucial to the degradation process. Water acts as the solvent for the pesticides and determines its availability for the microorganism. Dry soil tends to have slow biodegradation compared to wet soil. In water logged soil, anaerobic degradation has been

found to take place as opposed to aerobic degradation since there is limitation of oxygen entry to the soil. However, high moisture content may accelerate or hinder the degradation depending on the subject pesticide. On other hand, long term application of pesticides may have adverse effects on some of the soil physical chemical constituents. For instance, application of some of pesticides may lead to alteration of nitrogen (N_2) fixing organisms such as *Rhizobium*, *Azotobacter* and *Azospirillum* (Omakor *et al*., 2001). It may also affect cellulolytic and phosphate solubilizing microorganisms (Gulhane, Gomashe, and Sunderkar, 2015).

5.2 DHA activity and viable bacterial counts

Soil enzymes have been for long used as sensitive indicator of soil ecological disturbances in natural and agricultural ecosystems (Badiane *et al*., 2001; Sannino and Gianfreda, 2001). DHAs are key enzymes in the soil microbial respiratory processes and hence a good tool to assess microbial activity upon exposure of soil to pesticides (Cycoń, Piotrowska-Segetand, Kozdrój, 2010). According to Mambu (2014), DHA activity is higher in low doses application of pesticides and lower in high doses pesticides applied areas. In the case of current study, this could be one of the major reasons for the low overall DHA activity recorded in farm 139 and 212 untreated soil samples, which were on frequent application of the two herbicides compared to the higher activity recorded in farm OGF which had no history of herbicides use. The results from this study showed significant decrease in DHA activity across all farms upon treatment with 2,4-D as compared with untreated (control) soil samples. Hexazinone application boosted DHA activity in all the farms. The activity of the DHA enzymes was highly recorded in OGF farm in all experimental treatments. Initially upon the application of the two herbicides, 2,4-D and hexazinone, there was a lag phase in both pesticides, which took approximately two and half days. This could be attributed to the toxicity of the two herbicides towards the soil microorganisms. Pesticides cause respiration inhibition in their initial application stages. The effects are then recovered depending on whether the microorganisms are able to metabolize the respective compounds for their physiological needs (Monkiedje, 2006; Radivojević *et al*., 2008). Thereafter there was exponential increase in activity of DHA especially in hexazinone treated soils. This could have been due to recovery of microbial population and enzymes activity after initial inhibition due to microbial adaptation to these chemicals or due to their degradation thus being used by microorganisms as the source of carbon or nitrogen. Secondly this can also be due to microbial multiplication on increased supply of nutrients available in form of

microorganisms initially killed by herbicides as evidenced in other studies (Vandana, Rao and Padmaja, 2012; Latha and Gopal., 2010). According to Milosevia and Govedarica (2000), some microorganisms are able to metabolize herbicides immediately they are applied to the soil; however, there is secondary population of microbial community which may take a number of days before they can adapt to metabolize xenobiotics since enzymes responsible are inducible enzymes. The increase of DHA activity upon treatment of the soil with hexazinone was also supported by viable bacterial enumeration which showed that there was increased CFU compared to untreated soil sample. Study by Rahman *et al*. (2005) on impact of herbicide oxadiazon on microbial activity showed the herbicide was able to increase the enzyme activity in soil. Other studies that have shown the boosting ability of herbicides to soil microbial activity is that of Haney *et al*. (2000) and Araujo, Monterio, and Abarkeli, (2003) that showed glyphosate was able to increase soil microbial activity.

For 2,4-D treatments, the reducing effect of DHA activity was prolonged to approximately four days after which the activity was increased but at very low intensity as compared to the hexazinone treatment. The low DHA activity in 2,4-D treated soil samples was also supported by low viable bacterial density. These effects of 2,4-D to the DHA activity are similar with the findings by Mohiuddin and Mohammed (2014), who found that 2,4-D had inhibitory role on enzymes activity in soil for a period of 20 days and a decrease in inhibition of DHA was noted in 21st day following pesticide application. These findings also reported negative effects of 2,4-D on DHA activity in the initial application to control weeds in agricultural soils particularly in groundnuts cultivated soil (Hussain *et al*., 2009). The DHA activities recorded initially before the application of the two herbicides in this study were extremely high in OGF farm than in 212 and 139. This difference in DHA activity could be ascribed to the fact that soil enzymes activities are very sensitive to both natural and anthropogenic disturbances (Kumar *et al*., 2013). For instance, in this study, OGF farm was used as a control farm in which there was no history of herbicides application. Moreover, another factor which may contribute to the low DHA activity in 212 and 139 compared to the high activity in the untreated OGF farm is the regular use of tractor ploughing. This being one of the common anthropogenic disturbances experienced in farm 212 and 139, it may cause reduction in organic matter content in soil due to interference with the accumulation of crop residues in soil top layer and this may cause a reduction in microbial activity (Roldán *et al*., 2005). Soil microbial activity is more vigorous on soil rich in organic matter, and this could explain why there was high activity of DHA activity in OGF as compared to 212 and 139. Besides, herbicides decomposition also depends on the organic matter in the soil, this is

apparently because of vigorous microbial activities (Baboo *et al*., 2013). This factor can also explain why there was high activity of DHA in OGF farm when treated with hexazinone and 2,4-D as compared to other farms. However, the amount of the applied herbicides may also have a great effect on the amount degraded per a given time and also the residue effect of the herbicide detectable in soil (Ngigi *et al*., 2014).

5.3 Herbicides and metabolite residues

Herbicides residues analysis form the prerequisite for the biodegradation study. From the analysis the hexazinone residues obtained was higher compared to that obtained by Ngigi *et al*., (2014). However, Feng *et al*. (2008) reported hexazinone residue of 61 µg g^{-1} soil from soil. For 2,4-D and its metabolites 3,5-DCC the residues were 6.3±0.03 to 7.2±0.04 µg g^{-1} and 6.1±0.03 to 7.2±0.04 µg g^{-1} respectively. There are various factors that determine the persistence or the residue effects of herbicides in soil (Curran, 2016). For instance, the high level hexazinone and 2,4-D residues detected from this study could be due to their extensive use in an effort to increase sugarcane production and also high rate of application (Ngigi *et al*., 2014). Also, high residues detected from the study could be due to the time span of soil sample collection relative to herbicides application time. Moreover, hexazinone residue may be higher in soil with low pH as it was the case to our study of pH 4.9 and 5 (Curran, 2016).

5.4 Degradation of pesticides and molecular characterization of the bacterial isolates

Morphological, biochemical and molecular characterization of the hexazinone bacterial degraders revealed that N13010H1 was *Bacterium NLAE zl-H322* and N15030H2, N15030H3 and N212H4, being identified as *Enterobacter* sp, *Bacillus cereus* and *Staphylococcus aureus* strains, respectively. *Bacterium NLAE zl-H322* was first isolated from soil conatminated with crude-oil and showed ability to biodegrade hydrocarbons (Chikere and Ekwuabu, 2014). Though on the phylogenetic analysis N13010H1 clustered with *Enterococcus* sp, and despite it being reported with ability to biodegrade atrazine before, the biochemical test done in this case did not agree with those of *Enterococcus* sp and this led to the elimination of the isolate as *Enterococcus* sp. The tests on the other hand, coincided with those of *Bacterium NLAE* (Chikere and Ekwuabu, 2014). This finding could be reported as the first finding of the strain *Bacterium NLAE* capacity to biodegrade hexazinone compound. Several studies have reported biodegradation of atrazine by *Enterobacter cloacae.* El-Bestawy *et al*. (2013) reported a degradation capacity of 88.7% of the initial concentration used in their experiment. Other work that has reported degradation of hexazinone by

Enterobacter cloacae is by Ngigi *et al*. (2014), which reported 27.3% degradation capacity of initial concentration after an incubation of 28 days. Several studies have reported *Bacillus cereus* as a potential bioremedy for a number of xnobiotics. Przybulewska and Sienicka (2008), reported degradation of hexazinone by *B. cereus*. The strain has also been associated with biodegradation of petrolium based hydrocarbon and biosurfacant (Borah and Yadav, 2014). Other studies (Jayasri and Naidu, 2014) have reported ability of *B. cereus* to degrade herbicides such as profenofos and acetanilide herbicides with high efficiency. Though little work has been reported on bioremediation by *Staphylococcus* sp, Azizullah *et al*. (2014) reported *S. aureus* and other related species with ability to biodegrade atrazine from soil collected from Dera Ismail Khan District of Pakistan. In their study, the isolate was able to biodegrade the compound to 59.57% of the initial concentration. Their finding highly concide with the findings in this study in which 79.84% of hexazinone was biodegraded.

2,4-D bacterial isolates degraders N139D1, N13010D3 and N13010D4 were identified as *Bacillus* sp, *Serratia marcescens* and *Uncultured bacterium clone* respectively. *Bacillus* sp have been reported earlier in the degradation of wide range of chemicals like atrazine, chloropyrifos, metribuzin and imidacloprid (Jha *et al*., 2015). In their study Jha *et al*. showed, *Bacillus substilis* had a capacity to degrade the four xenobiotics by 60, 60, 25.41, and 32.13 percent respectively. In his work on laboratory analysis of *tfd A* gene involvement in degradation of 2,4-D, Han *et al*. (2014) reported degradation of 2,4-D by 50-80% of the initial available concetration, findings which are consistent with current study whereby *Serratia mercescen* degraded 2,4-D by 65.2% , *Uncultured bacterium clone* by 74.3% and *Baccilus sp* 82.3% in 28 days. *Bacillus* sp has shown potential to biodegrade 2,4-dichlorophenol (2,4-DCP) and was reported by Wang *et al*. (2000) in their study on bacterial degradation of chlorophenols and their derivatives (Arora, and Bae, 2014). Silva *et al*. (2007) reported *Serratia marcescens* ability to biodegrade 2,4-D from Brazilian soil contaminated with the herbicide which was the first study to show the ability of *Serratia marcescens* to degrade 2,4-D. The bacterium has also been associated in the biodegradation of glyphosate (Benslama and Boulahrouf, 2013) and dibenzofuran (Jaiswal *et al*., 2011).

CHAPTER SIX
CONCLUSION AND RECCOMENDATIONS

6.1 Conclusion

Most of the analyzed physicochemical parameters (P, K, Mg and N) were very low. In addition, soils from all the fields were found to be acidic. There were high residues of hexazinone and 2,4-D and it metabolite (3,5-DCC) detected from the soil samples. This raises concern on the recommendable amount for the two herbicides that should be applied to control weed. Also, the high amounts of residues detected from the study poses threat to the human and other non-targeted organism due to the herbicide adverse effects on environment.

From the findings of this study it can be concluded that 2,4-D application has a negative impacts to the general soil mocrobial activity as evidented by low dehydrogenase enzymes activity and low bacterial density in the experiments while hexazinone has no negative effects at its field application rate as evidented by higher dehydrogenase enzymes activity and bacterial density in the experiment. This finding therefore, raises concern on the appropriate field rate at which the 2,4-D should be applied as well as its potential sub lethal effects. Although the use of herbicides is important as it offers less expensive and effective way of weed control, their application should be considered due to the detrimental effects they pose to the untargeted organisms. This study also reinforces the use of DHA as a quick and reliable method to determine effects of pesticides on soil microorganisms.

There were various bacterial degraders of the two xenobiotics isolated from this study, which are *Enterobacter* sp, *Bacterium NLAE zl-H322, Staphylococcus aureus* and *Bacillus cereus* for hexazinone and *Serratia marcescens, Bacillus* sp and *Uncultured bacterium clone* for 2,4-D. The bacteria isolates were also found to exhibit different rates of biodegradation for the two herbicides and showed synergism in their consortium compared to pure isolates. These finding are good insights for the bioremediation work that there are such biodegraders in Nzoia soils.

6.2 Recommendations

1. More investigations need to be done on identified microorganisms, especially on the in-situ biodegradation and complete elucidation of biodegradation.
2. Synergism mechanisms of the microorganism in biodegradation of the two herbicides as well as mechanism and pathways need to be further investigated.
3. There is a need to carry out the study on the toxicity dose of the two herbicides on the soil microorganism as indicated by DHA activity.

4. Continuous monitoring of residual herbicides should be done and their fate in soils established, such as biodegradation.

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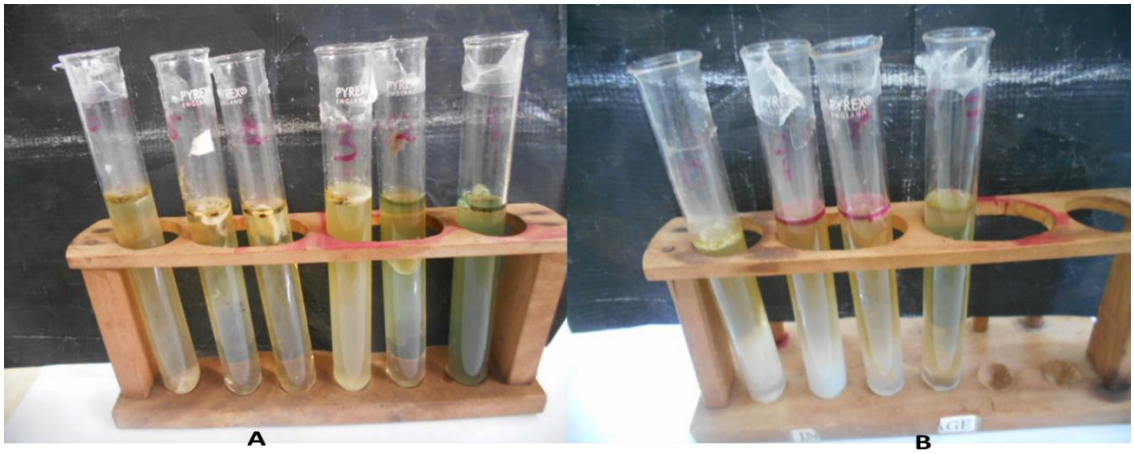
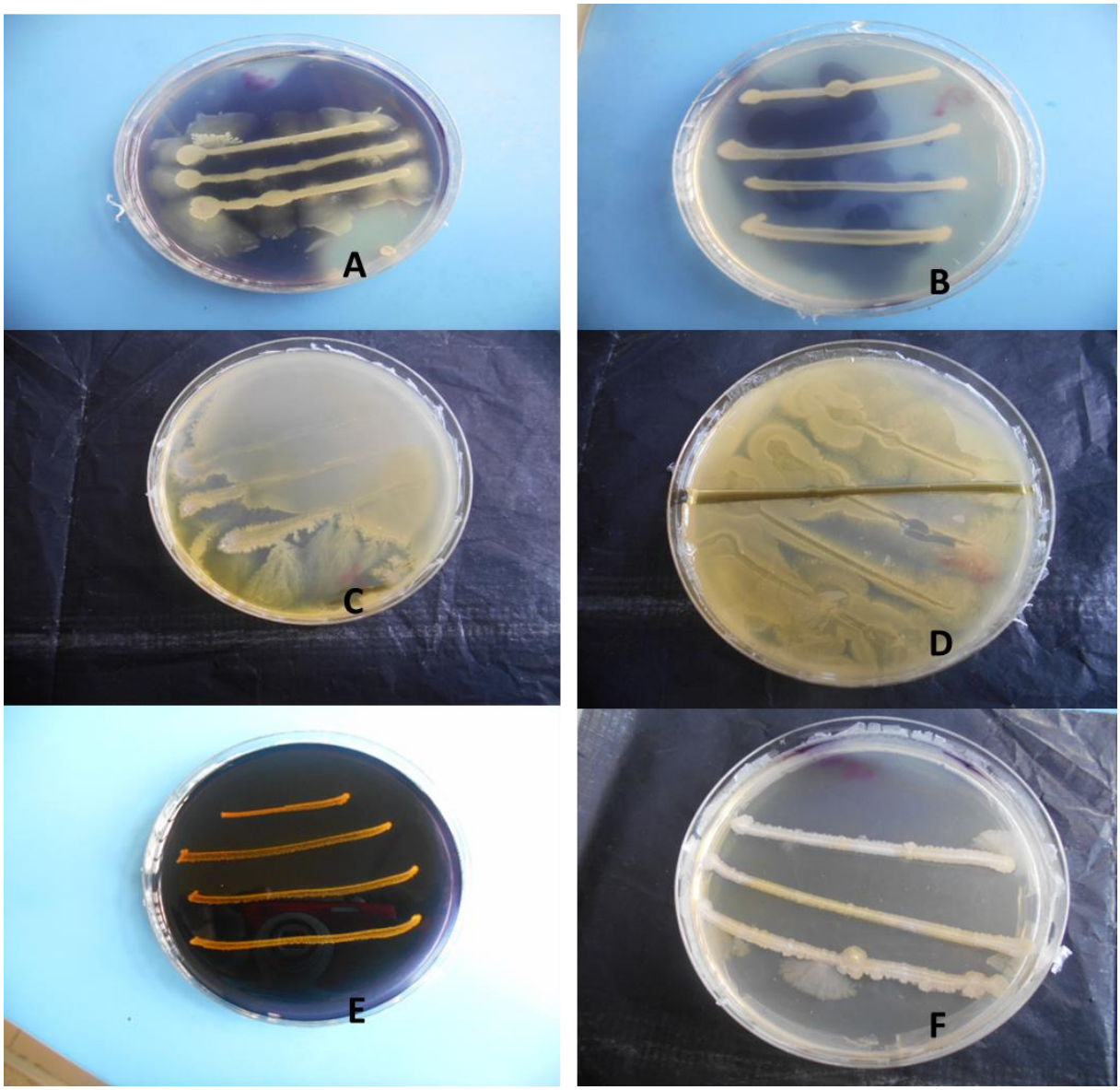
APPENDICES

Appendix 1; Means table and std. Dev of TPF concentrations for the three farms within seven days period. Means followed by the same letter in the same column are not significantly different at 5% LSD

		Treatments					
		Control		**Hexazinone**		**2,4-D**	
		TFF Conc		**TPF Conc**		**TPF Conc**	
Farms	**Days**	**(µg g^{-1} soil)**	**Std.Dev**	**(µg g^{-1} soil)**	**Std.Dev**	**(µg g^{-1} soil)**	**Std.Dev**
	1	3.199^{d}	±0.134	1.794^{e}	±0.134	-0.817^{c}	±0.134
	2	5.408^{c}	±0.134	1.727^{e}	±0.116	0.522^{b}	±0.116
	3	5.716^{c}	±0.140	17.189^{d}	±0.232	0.656^{ab}	±0.134
212	4	5.877^{c}	±0.177	20.736^{c}	±1.161	0.790^{ab}	±0.177
	5	6.814^{b}	±0.177	29.237^{b}	±0.116	0.656^{ab}	±0.134
	6	7.617^{a}	±0.241	41.486^{a}	±0.232	1.058^{ab}	±0.177
	7	8.086^{a}	±0.177	41.620^{a}	±0.067	0.924^{a}	±0.232
	1	1.058^{c}	±0.134	4.003^{e}	±0.373	0.120^{c}	±0.116
	2	4.070^{b}	±0.177	9.290^{de}	±0.241	0.254^{bc}	±0.177
	3	4.137^{b}	±0.116	15.067^{dc}	±1.101	0.321^{bc}	±0.000
139	4	4.337^{b}	±0.116	18.099^{bc}	±0.466	0.522^{abc}	±0.116
	5	4.538^{b}	±0.116	18.059^{bc}	±0.745	0.321^{bc}	±0.116
	6	6.680^{a}	±0.469	26.426^{a}	±6.127	1.191^{a}	±0.354
	7	6.881^{a}	±0.177	23.681^{ab}	±3.180	0.991^{ab}	±0.572
	1	5.810^{f}	±0.292	6.546^{e}	±0.116	0.054^{f}	±0.177
	2	8.153^{e}	±0.232	10.964^{d}	±0.116	1.392^{e}	±0.177
	3	8.286^{e}	±0.067	114.043^{c}	±2.377	1.995^{d}	±0.067
OGF	4	15.917^{d}	±0.177	144.632^{b}	±0.639	5.609^{c}	±0.177
	5	31.780^{c}	±0.134	172.477^{a}	±0.177	11.700^{b}	±0.241
	6	39.813^{b}	±0.839	172.544^{a}	±0.268	12.503^{a}	±0.134
	7	41.285^{a}	±0.116	172.945^{a}	±0.595	12.905^{a}	±0.067

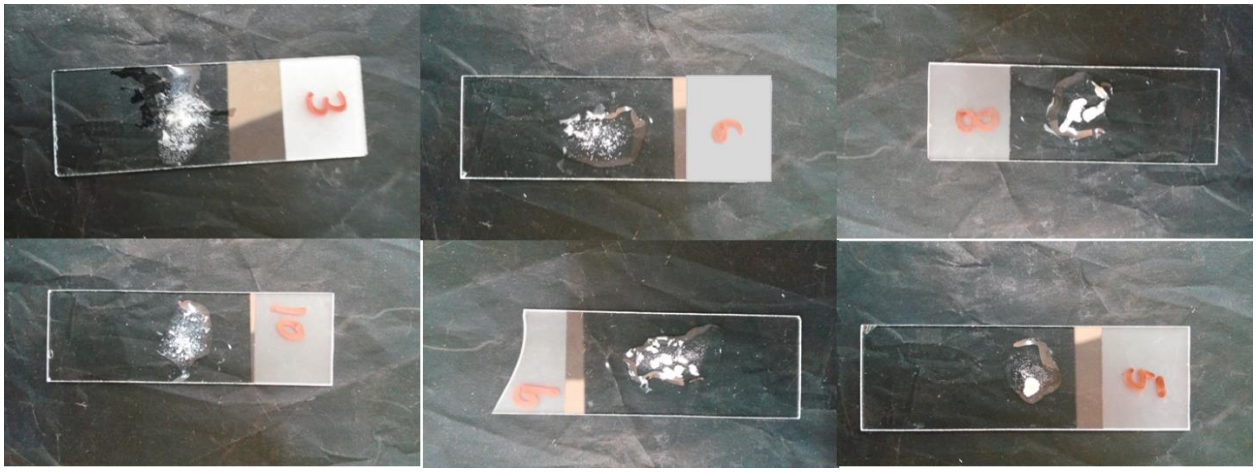


Appendix 2: Indole test for the four isolates and two reference bacteria (A) Tube number 1 (N139D1), 3 (N15030H3), 5 (*B-spizizenii*) and 6 (N15030H2) all showing negative results for indole test. (B) Tube number 7 (N13010D3), 8 (N13010H1), and 9 (*E. coli*) and 10 (N212H4) with tube 8 and 9 showing positive results and tub 7 a and tube 10 showing negative result for indole test.

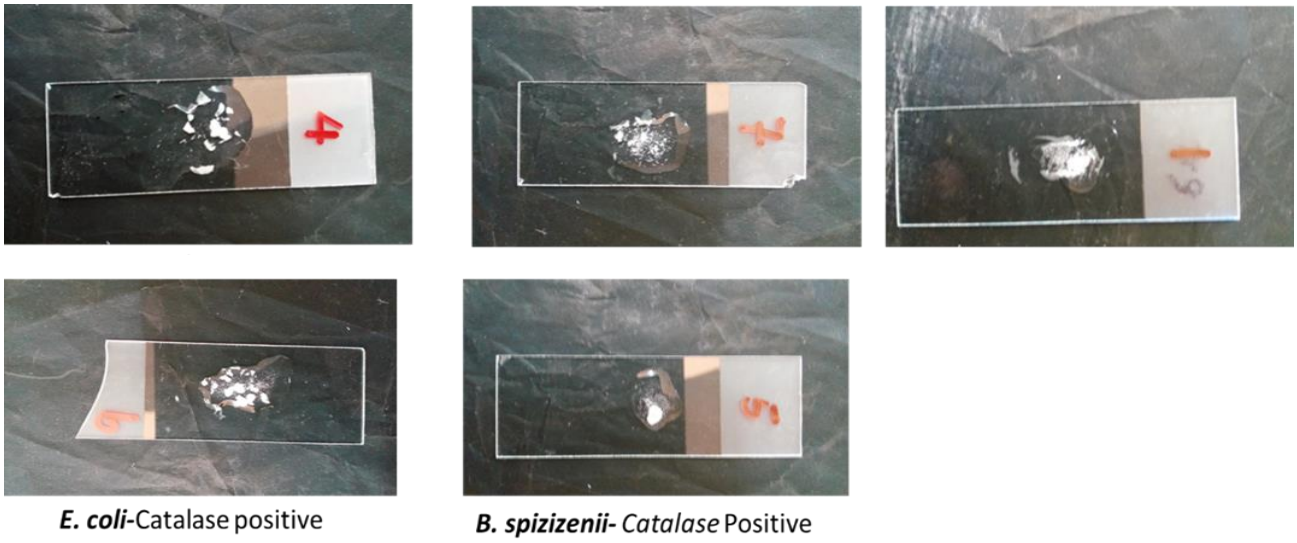


Appendix 3: Starch hydrolysis test for the four isolates and two reference bacteria (A) N13010H1-Negative (B) N15030H2-Negative (C)N15030H3-Negative (D) N212H4-Positive (E) *E. coli*-Negative and (F) *B. spizizenii*-positive.

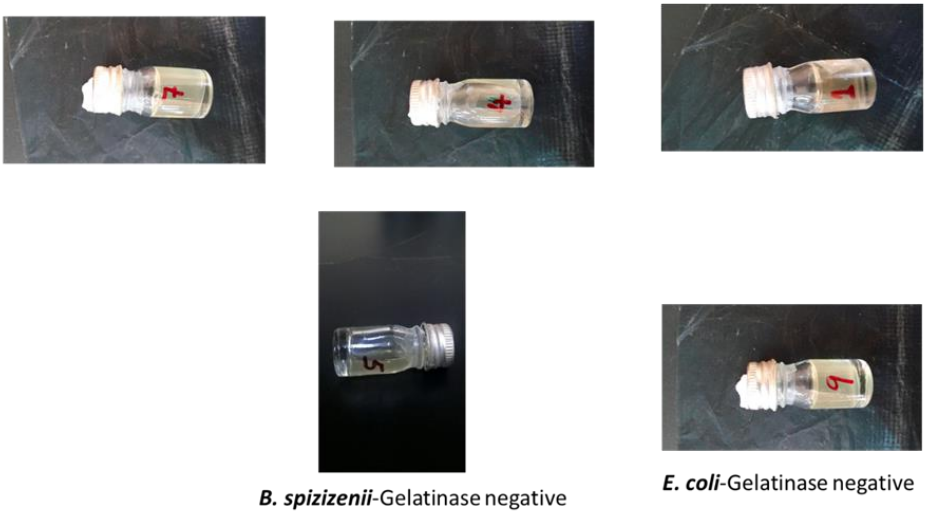
Appendix 4: Gelatin hydrolysis tests: 3-(N15030H3)-Positive, 6 (N15030H2)-Negative, 8 (N212H4)-Negative, 10 (N212H4)-Negative, 5 (*B. spizizenii*)-Positive and 9 (*E. coli*)-Negative.



Appendix 5: Catalase test for the four isolate and the reference bacteria. 3-(N15030H3)- Positive, 6 (N15030H2)- Positive, 8 (N212H4)-Negative, 10 (N212H4)- Positive, 5 (*B. spizizenii*)- Positive and 9 (*E. coli*)- Positive.



Appendix 6: Catalase tests for 4-N139D1,7- N13010D3 and 1-N13010D4 isolates and two known bacterial reference cultures



Appendix 7: Gelatinase tests for 4-N139D1, 1-N13010D3, and 7-N13010D4 isolates and two known bacterial reference cultures