ISOLATION AND CHARACTERIZATION OF SOIL BACTERIA CAPABLE OF DEGRADING METRIBUZIN IN SUGARCANE FARMS OF WESTERN KENYA

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DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this is my original work and has not been presented in this or any university for the award of a degree

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DEDICATION

This thesis is dedicated to my husband Mac, my daughter Ray and son Ian.

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ABSTRACT

Nzoia River Drainage Basin is a major sugar production region in Kenya. Various pesticides are applied in this area to control weeds and boost sugar productivity. However, use of herbicides for weed control leads to increased chemical loads in the environment whose effects could be disastrous to the biotic component. The use of pesticide adapted microorganisms in the degradation and detoxification of many toxic xenobiotics, especially pesticides, is an efficient tool for the decontamination of polluted environments. The main objective of this work wasto isolate and identify soil bacteria capable of degrading metribuzin, a commonly used weed killer in sugarcane farms.Five farms with history of metribuzin application were purposively identified for soil sampling. Random soil sampling was used to obtain samples from a depth of 0 – 10cm in November 2013. A composite sample was used in isolation of the bacteria. Influence of temperature, pH, nitrogen and phosphorous on growth of the isolated bacteria was also tested. Experimental design was used to carry out the experiments in the laboratory. Mineral salts media containing metribuzin as the sole carbon source was used to culture and selectively isolate metribuzin degrading bacteria. Growth of the bacteria in the medium measured as absorbance at OD_{600}nmfor various time intervals was an indication of tolerance to the herbicide and ability to utilize metribuzin as a carbon source. HPLC method was used to determine ability of the bacteria to degrade metribuzin and assess the metabolites after the 21 day incubation period. Molecular analysis was carried out by DNA extraction from each isolate and subjected to PCR using 16S primers. Sequences and blast results were compared to relevant data bases. ANOVA and separation of means using LSD at $p \leq 0.05$ was used to analyse data. Seven different bacteria isolates with metribuzin degrading potential were coded NZ453A, NZ454B, NZ453C, NZ543A, NZ543B, NZ8070 and NZ1110. They were subjected to morphological, cultural, biochemical and molecular characterization. Results also revealed thattemperature, pH, nitrogen and phosphorous had different influence on the specific bacteria but generally, 35^{o}C, pH 9, nitrogen and phosphorous concentrations of 7.5g/L recorded highest growth on most of the isolates. The isolates degraded more than 93% of metribuzin. The seven isolated bacteria were identified as *Planococcus sp., Burkholderia cepacia, Pseudomonas sp., Bacillus sp., Arthrobacter sp., and Staphylococcus sp.,* all of which have been previously associated with degradation of recalcitrant compounds in the environment. This indicates that Nzoia sugarcane farms consist of different metribuzin degrading bacteria, which can grow in different physical chemical conditions. They can be multiplied and further developed for bioremediation or bioaugmentation of metribuzin contaminated sites.

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LIST OF ABBREVIATIONS AND ACRONYMS

CCME	Canadian Council of Ministers of the Environment.
DA	Desamino
DADK	Desamino-diketo
DK	Diketo
EXTOXNET	Extension Toxicological Network.
HPLC	High Performance Liquid Chromatography.
HSDB	Hazardous Substances DataBank.
KESREF	Kenya Sugar Research Foundation.
NRDB	Nzoia River Drainage Basin.
NSF	Nzoia Sugar Farm
OMAF	Ontario Ministry of Agriculture and Food
OMOE	Ontario Ministry of Environment.
PCR	Polymerase Chain Reaction.
RTECS	Registry of Toxic Effects of Chemical Substances
USEPA	United States Environmental Protection Agency

CHAPTER ONE
INTRODUCTION

1.1 Background information

Sugarcane is an important cash crop in Kenya and it is the major economic activity undertaken in Western Kenya among other agricultural activities (KESREF, 2009). Weeds in sugarcane growing areas are a major challenge to cane production and can reduce sugar yields significantly as they compete for moisture, nutrients, and light during the growing season as well as acting as alternate hosts for disease and insect pests. Heavy weed infestations can also interfere with sugarcane harvest by adding unnecessary harvesting expenses and can act as source of seed bank replenishment and re-infestation in subsequent years (KESREF, 2009). Herbicides such as atrazine (as Gesapaxcombi), Metribuzin, hexazinone, diuron, actril DS (isoxynil + 2,4-D ester), ametryn and glyphosate are frequently used for controlling grasses and broad leaf weed in different crop fields (KESREF, 2009).

The excessive use of herbicides leads to accumulation of a huge amount of residues in the environment, thereby posing a substantial health hazard for the current and future generations due to uptake and accumulation of these toxic compounds in the food chain and drinking water (Monisha *et al.,* 2014). Pesticides applied on land subsequently find their way into water bodies through runoff causing trans- media pollution. In deed residual pesticides have been detected in soil and water in the Nzoia River Drainage Basin (NRDB) in Kenya (Getenga *et al*., 2004, Ngigi *et al*., 2011).

Long exposure of the soils to triazine and chlorophenoxy-herbicides that are used in Kenyan sugarcane farms may have resulted in the adaption of the native soil microbes leading to enhanced degradation (mineralization) of the herbicides in the soil (Ramdas and Sims, 2011). Studies conducted in Kenya and other parts of the world show that adapted microbes indeed do exist in the soils with long exposure to the herbicides (Abdelhafid *et al*., 2000; Getenga *et al*., 2009; Ngigi *et al*., 2011). Microbes showing enhanced herbicide degradation abilities can be used in biogumentation experiments to stimulate degradation of herbicides in contaminated fields (Rousseaux *et al*., 2003; Aislabie *et al*., 2005).

Metribuzin is a herbicide in the group of triazinones and it is listed as a toxic release inventory (TRI) chemical (USEPA, 1996). It is moderately adsorbed on soils with high clay or organic content (HSDB, 2000) The half-life of metribuzin increases with decreasing soil

moisture content, depth, temperature and pH (Malone *et al.,* 2004). Microbial metabolism is the major pathway for the removal of metribuzin from soil. This study screened soils from sugarcane farms of Nzoia Sugar Company, in NRDB for bacteria with metribuzin degrading potential. The findings from this study may be useful for bioremediation of metribuzin contaminated soils in generating information that may be used in subsequent similar studies.

1.2 Statement of the problem

Pesticides have played an important role in the increase of agricultural production for food security to an ever increasing world population. The range of damage associated with the application of pesticides to the environment and different receptors is great. The major environmental impacts of pesticide use in agriculture include; loss of aquatic and terrestrial biodiversity, contamination of surface and ground water, agricultural produce and poisoning of agricultural workers among other potential negative impacts. Metribuzin is a widely preferred herbicide because of relatively low cost, unrestricted use and is considered for use more often, after banning of residual pesticides in most countries. It has been detected in both surface and ground water, in areas of use in different studies. It is a frequently used herbicide in sugarcane farms of Western Kenya.

Recent studies in Kenya have indicated the presence of pesticide residues in sugarcane cultivated soils and water from the drainage basins. As a result, there is growing concerns regarding the potential effects of pesticides on non-target organisms such as soil microbes, man, and livestock among others. Erosion of sugarcane soils and subsequent transport of sediment-bound contaminants in river runoffs into Lake Victoria is a growing concern as the sugar industry continues to expand. Lake Victoria is an important natural resource to the East African region and beyond in the fishing industry, farming, source of domestic water supply, transportation and source of river Nile, which is also the main source of livelihoods for North African countries. Available animal studies suggest metribuzin exposure causes low acute toxicity. Sub-chronic studies suggest that metribuzin could cause adverse effects in body weight gain, organ weight and haematological parameters.

1.3 Objectives

1.3.1 General objective

To assess the presence and physical chemical growth conditions of potential metribuzin degrading bacteria in Nzoia sugar belt farms, for probable use in bioremediation.

1.3.2 Specific objectives

1. To determine bacteria population distribution, isolate and characterize bacteria with potential of degrading metribuzin in Nzoia sugar company farms.
2. To determine the influence of temperature, pH, phosphorous and Nitrogen on growth of metribuzin degrading isolates.
3. To determine degradation potential and molecular identities of metribuzin degrading bacteria.

1.4 Hypotheses

1. There is no significant difference in soil bacteria population distribution and bacteria isolates with potential of degrading metribuzin in Nzoia sugar company farms.
2. There is no significant difference in growth of the isolates due to effects of temperature, pH, phosphorous and nitrogen.
3. There is no significant difference in degradation potential and molecular identities of metribuzin degrading bacteria.

1.5 Justification

In Kenya large amounts of metribuzin are used to control weeds in sugar belts of Nzoia, Mumias and Sony, as well as wheat and maize farming in Eldoret, tea farming and coffee plantations in the country. Despite the herbicide's importance as a herbicide, it is known to have a half-life of between two and a half months to four months in soils and is a surface and ground water contaminant. It is also known to have negative health effects, for example internal organs enlargement and haematological conditions, in animals after ingestion. Microbial degradation is the principle route of removal of metribuzin and it is considered an environmental friendly approach in pollution management. Bioremediation which includes the gainful utilization of effective microorganism for the metabolism or biodegradation of target pollutants into safer and innocuous products is amongst the potent technologies that are being used globally for the restoration of contaminated sites. A contemporary approach in this regard is the utilization of microorganisms especially bacteria due to fast growth and metabolism. Bacteria are well known for their diversity and versatility with regard to both physical and chemical environments. Many bacteria that are able to degrade pesticides have been isolated from soil around the world. In Kenya, the presence and the role of metribuzin degrading bacteria in soil has not been demonstrated in agricultural systems, even though studies show metribuzin presence in natural waters where the herbicide is used. The growth conditions, bacteria distribution and influence of physico-chemical

parameters on bacteria populations are also not known hence there is need to establish multiplication conditions of the bacteria for essential uses which include bioremediation. This study will contribute to acquisition of new knowledge in research, increased government database for use in policy formulations, in herbicide use and will create awareness among the nuclear estate managers about the effects of herbicides to the environment and bioremediation methods in restoration of contaminated sites.

1.6 Scope of the study

Among the many farms, soil samples were obtained purposively from five farms with history of metribuzin application within a period of two to three years at the time of sampling. It is within this duration that metribuzin was used frequently as per data from the Nzoia Sugar Company. The farms were part of Nzoia Sugar Company Nucleus estate farms with designated numbers and were representative of farms with metribuzin treatment. Among many other microorganisms known to degrade xenobiotic compounds, this study researched on bacteria in regard to their versatility and fast metabolism. This study was done *in vitro*asone of the most appropriate method of isolating and identifying specific degraders with limited resources for the study.Other herbicides were used in the study area but Metribuzin was studied because it has been detected as a residue in Nzoia River Drainage Basin, as reported in other studies (Ngigi *et al.,* 2011). It is also frequently used as an alternate herbicideto other herbicides.

1.7 Assumptions

It was assumed that the genera of metribuzin degrading bacteria present were not affected by variations in weather, such that they were present in the soil in all seasons, although sampling was done during the rainy season. The bacteria were assumed to be adapted to degrading metribuzin *insitu* and their multiplication *invitro* could be successfully used for bioaugmentation of contaminated sites in the field.

1.8 Definition of terms

Pesticide

Any chemical substance used for killing pests, as insects, weeds etc. (Anon, 1999)

Herbicide

Also commonly known as weed killers, are pesticides used to kill unwanted plants (Anon, 1999).

Xenobiotic compound

These are chemicals which are foreign to the biosphere**.**

Bioremediation

Bioremediation is a process that uses mainly microorganisms, plants, or microbial or plant enzymes to detoxify contaminants in the soil and other environments (Hance 1991).

Biodegradation

Biodegradation refers to the partial, and sometimes total, transformation or detoxification of contaminants by microorganisms and plants (Hance 1991).

Metribuzin degrading isolates

These refer to bacteria that were isolated from the soil samples, which utilized metribuzin as the sole carbon source, thus breaking down the metribuzin compound into metabolites.

CHAPTER TWO
LITERATURE REVIEW

2.1 Importance of sugarcane farming

Sugarcane (*Saccharum sp.*) is one of the most important crops in the world because of its strategic position and immense uses in the daily life of any nation as well as for industrial use, aimed at nutritional and economic sustenance. Sugarcane contributes about 60 % of the total world sugar requirement while the remaining 40 % come from sugar beet (Onwueme and Sinha, 1999). It is a tropical crop that usually takes between 8-12 months to reach its maturity. The important sugar-producing countries in the tropical Africa are Mauritius, Kenya, Sudan, Zimbabwe, Madagascar, Cote d'Ivoire, Ethiopia, Malawi, Zambia, Tanzania, Nigeria, Cameroon and Zaire, (NSDC, 2003).

2.2 Economics of sugar industry in Kenya

The sugar industry in Kenya is a major contributor to the agricultural sector which is the mainstay of the economy and supports livelihoods of at least 25 % of the Kenyan population (Mitchell, 2004). The subsector accounts for about 15 % of the agricultural GDP, is the dominant employer and source of livelihoods for most households in Western Kenya comprising Nyanza, Rift Valley and western provinces (Mitchell, 2004). The industry saves Kenya in excess USD 250 million (about 19.3 billion) in foreign exchange annually and contributes to tax revenues to the exchequer (KESREF, 2007).

Sugarcane production costs in the Kenyan sugar industry include such inputs as land preparation, acquisition of seed cane, fertilizers and herbicides, labour costs and cane transport to the factories. These costs have been increasing over time, in addition to processing costs. The result is high domestic sugar price in comparison to other sugar producing countries (Mitchell, 2004).

2.3 Sugarcane production

Sugarcane production is faced by many constraints that hinder production. Like any other crop, sugarcane is susceptible to attack by many diseases and insect pests, nematodes and weeds. Weeds in sugarcane growing areas are a major challenge to cane production and can reduce sugarcane yields by more than 20-25% (Khan *et al*., 2004) as they compete for moisture, nutrients, and light during the growing season as well as acting as alternate hosts for disease and insect pests. Heavy weed infestations can also interfere with sugarcane

harvest by adding unnecessary harvesting expenses, and can act as source of seed bank replenishment and re-infestation in subsequent years (Odero and Dusky, 2009). The major weeds of sugarcane producing zones comprise the broad leaved plants such as *Striga, Amaranthus*, *Datura* and *Tagetes* species, and the narrowed leaved types, such as, Couch and Johnson Nutsedge. Bermuda grasses and these weeds can cause between 30 and 100% loss in yield in the sugar growing areas of Kenya (KESREF, 2007). Various pesticides used to control weeds in sugarcane production systems worldwide, are described by Odero and Dusky, (2009). They include pesticides that are used in Kenya to control weeds. Some of the pesticides used in Nzoia sugar belt are glyphosate, atrazine (as Gesapaxcombi), hexazinone, diuron, actril DS (isoxynil + 2, 4-D ester), ametryn, metribuzin and lasso (KESREF, 2007)

2.4 Metribuzin as a pesticide

Metribuzin ($C_8H_{14}N_4OS$) is a selective triazinone herbicide used for broadleaf and grass weed control in various crops, (OMAF, 1988). Common uses include application to soy beans, potatoes, alfafa, sugarcane, barley and tomatoes (USEPA, 1998; Larson *et al.*, 1999). Metribuzin is listed as a toxic release inventory (TRI) chemical (USEPA, 1996). It is released into the environment primarily during agricultural spraying operations. Studies reported by HSDB (2000) show that; metribuzin is moderately adsorbed on soils with high clay or organic content, its adsorption decreases with increasing soil pH, little leaching occur in soils with a high organic content, but it is readily leached in sandy soils. Whereas some non-biological degradation occurs, microbial metabolism is the major pathway for the removal of metribuzin from soil.

2.5 Physical and chemical properties of metribuzin

Metribuzin is a white crystalline solid with a melting point of 126 °C. Pure metribuzin is soluble in water up to 1,200 ppm ($1.2gL^{-1}$). Metribuzin has a slight sulphurous odour. It is reported to have a vapor pressure of between 5 and 10 mm Hg at 20°C and a density of 1.31 g/cm^3between 4 and 20°C (USEPA, 1998; HSDB, 2000).The common name is Metribuzin and chemical name is 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H) one. The names and synonyms of metribuzin are;Lexone, Lexone DF, Lexone 4LMetribuzine, Sencor, Sencor 4, Sencoral, Sencor DF, Sencorer, Sencorex , Sengoral and Zenkor, (USEPA, 1998; RTECS, 2000; HSDB, 2000). The chemical structure of the compound is as illustrated in Figure 1.

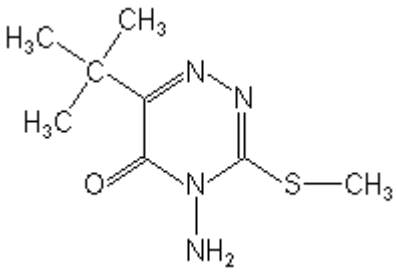


Figure 1: Chemical structure of metribuzin.

2.6 Metribuzin in the environment

Metribuzin is a persistent herbicide and studies have shown that residual amounts can range between 0 to 20 % in soil environment (Smith, 1982). Studies have indicated that the optimal pH for maximum adsorption of metribuzin to soil colloids is between pH 4.0 and 5.0 (Rigi *et al.,* 2015). In other soil studies metribuzin was found to be more mobile in coarse soils and the adsorption coefficients for various soils were negatively correlated with the mobility (Daniel *et al.,* 2002). The study reported that the only soil property significantly correlated with herbicide behaviour was sand content, with mobility being increased and phytotoxicity being diminished by increased sand content. Soil environment can dramatically affect degradation of pollutants. Environmental conditions may not always be the optimal for growth, or enzyme production by fungi and bacteria, but for pollutant transformation (Singleton, 2001). The fluctuating conditions affect degradation of metribuzin as well as other pollutants. Studies show that photo degradation and volatilization influence the loss of metribuzin (Jensen *et al*., 1989). Half-life values of 4–5 d have been reported as calculated for metribuzin on the soil surface when exposed to "warm" temperatures and intense irradiation (Savage, 1980). Photochemical degradation was found to proceed only in the presence of moisture (Bartl and Korte, 1975).

Pesticides enter aquatic ecosystems through surface runoff after intensive irrigation or heavy rainfall, and as a consequence have become some of the most frequent organic pollutants in aquatic ecosystems (Pintel, 1995). Metribuzin is highly soluble in water and it has a moderate ability to adsorb to soil with high clay and/or organic matter content so it has great potential to leach into, and contaminate groundwater (EXTOXNET, 1993). Soil half-lives of less than one month to six months have been reported (EXTOXNET, 1998). Others studies have shown that metribuzin can remain for a longer time in soil (until 377days under aerobic conditions) depending on temperature, pH and soil type, and accumulate in plants and interfere with other crops (EXTOXNET, 1993).

2.7 Effects of metribuzin on health

Studies relating to the metribuzin exposure on human health effects are lacking (USEPA, 2003) and therefore, hazard characterization are performed from the available animal studies. Studies conducted in animals suggest that metribuzin exposure causes low acute toxicity as evidenced by the reported high LD_{50} values (Kimmerle *et al*., 1969; Morgan, 1982; Hartley and Kidd, 1987). Also, acute exposure studies suggest that metribuzin, at the doses tested do not result in eye or dermal irritations (Kimmerle *et al*., 1969). Sub-chronic studies suggest that metribuzin could cause adverse effects in body weight gain, organ weight, and haematological parameters. For example, a significant reduction in body weight gain and an increase in liver and thyroid weights were reported in Wistar rats exposed to metribuzin at 1,500 ppm (Loser *et al*., 1969). Three weeks of dermal exposure to metribuzin (1,000 $mgkg^{-1}$) in rabbits also resulted in an increase in liver enzymes such as N-demethylase and cytochrome P450 (Flucke and Hartmann, 1989). These effects are not pronounced when the studies were conducted at lower doses in dogs. Three-month metribuzin exposure to Beagle dogs did not affect body weight gain or food consumption, but altered some clinical parameters (Chaisson and Cueto, 1970).

Chronic effects of metribuzin exposure may include changes in body weight gain, mortality, liver enzyme activities and histopathological changes. Two-year feeding studies were performed on rats (Christenson and Wahle, 1993), mice (Hayes, 1981) and Beagle dogs (Loser and Mirea, 1974). In general, there were no significant differences in body weight gain, food consumption or mortality after years of exposure to metribuzin to rats (Chiali *et al.,* 2013) and mice (Hayes, 1981). However, Christenson and Wahle (1993) observed a decrease in body weight gain in rats after metribuzin treatment. Major histo-pathological changes reported by one study after chronic feeding of metribuzin include a significant increase in corneal neovascularization, the incidence of a discoloured zone in the liver, an enlarged abdomen, enlarged adrenal and thyroid glands, ocular opacity, and enlarged epididymal mass in male rats and the presence of ovarian cysts in female rats (Christenson and Wahle, 1993).

2.8 Microbial degradation of pesticides and growth conditions

The use of microorganisms in the degradation and detoxification of many toxic xenobiotics, especially pesticides, is an efficient way of the decontamination of polluted sites in the environment (Mohammed, 2009). When pesticides are released in the environment

their fate is decided by various environmental conditions and microbial degradation (Bhadbhade *et al.*, 2002). Microorganisms are the primary agents of biological recycling, and have evolved an extensive range of enzymes, pathways and control mechanisms in order to degrade and utilise pollutants as energy sources (Madigan *et al.*, 2000, Talaro and Talaro, 2002). In soils, one likely scenario for the complete degradation of herbicides may be the initial enzymatic attack by relatively nonspecific oxidases (such as the peroxidases produced by fungi and some actinomycetes), followed by further metabolism by hydrolases and/or ring cleavage enzymes, eventually resulting in products which are mineralized via catabolic pathways. Consequently, an effective bioremediation strategy for herbicides might include the stimulation of and/or inoculation with oxidase producing microorganisms in order to accomplish the partial degradation of the parent compound to products that are degradable by other microbes (Madigan *et al.*, 2000; Talaro and Talaro, 2002).

Long exposure of the soils to triazine and chlorophenoxy herbicides among others, has resulted in the adaption of the native microbes resulting in enhanced degradation (mineralization) of the herbicides in the soil. Studies conducted in Kenya and other parts of the world show that adapted microbes indeed do exist in the soils with long exposure to the herbicides (Abdelhafid *et al*., 2000; Getenga *et al*., 2009; Ngigi *et al*., 2011). It has also been found that soils without adapted micro flora to enhanced herbicide mineralization have been stimulated by bioaugmentation whereby indigenous microorganisms that are capable of catabolizing the herbicide are added to soils to enhance the degradation of the chemical (Rousseaux *et al*., 2003; Aislabie *et al*., 2005).

Microbial degradation is governed by many factors but the most critical ones being soil temperature, soil moisture content and soil pH. A temperature range of 25^{o}C to 35^{o}C and a soil moisture range of 50% to 100% of field capacity are considered optimum for the action of microbes on herbicides (Rao, 2000). The effect of soil pH on microbial degradation of herbicides is highly specific depending on the herbicides and microorganisms involved (Singh 2008). Generally, bacteria predominate in alkaline soils, with greatest total microbial life in soils in neutral pH. Temperature directly affects the activity of the soil biota by determining the rate of physiological activity such as enzyme activity and indirectly by affecting physico-chemical properties such as diffusion and solubility of nutrients. pH directly affects the solubility of elements. Essential minerals can become unavailable at extremes of pH. Soil moisture affects the soil biota in two ways. Biologically water is

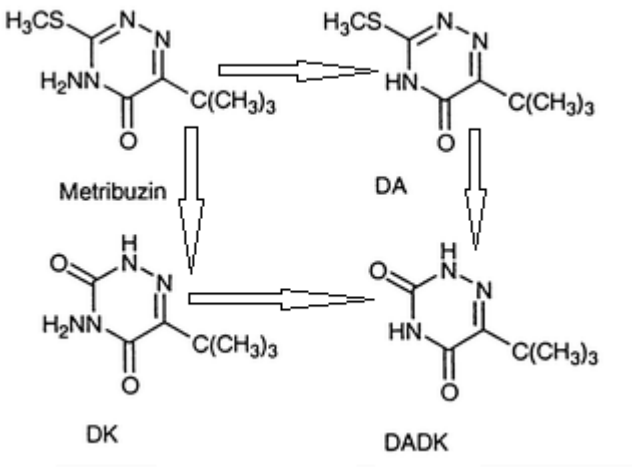
essential for life and for enzyme activity and metabolism and, is a solvent for biological nutrients and other chemicals. Physically, soil moisture affects soil temperature (water is good conductor of heat) and soil aeration. The degree at which soil pores are filled affects the movement and predation of microorganisms in soil. The predominant genera of herbicide degradation are; *Agrobacterium, Achromobacterium, Alcalaginese, Arthrobacter, Bacillus, Flavobacterium, Pseudomonas, Norcadia, Streptomyces, Rhizobium* and others (Rao, 2000). During degradation, microorganisms use carbon atoms of the herbicide particle for their energy requirement (Killham, 1994).

2.9 Fate of metribuzin degradation

Metribuzin is one of the herbicides frequently used to manage weeds in sugar farms and has received a great deal of attention in other parts of the world because of its extensive use and potential for widespread contamination of ecosystems (Lawrence *et al.*, 1993). The fate of this pesticide has been extensively studied under aerobic conditions (Pavel *et al.*, 1999). Although some non-biological degradation occurs, (Webster *et al*. 1978), microbial metabolism is the major pathway for the removal and loss of metribuzin from soil; (Ashim, *et al.*, 2008) and factors that influence microbial activity such as temperature, moisture and nutrient levels influence its degradation (Sharom and Stephenson, 1976). Metribuzin is degraded to carbon dioxide in soil. Loss from soil surfaces by photodecomposition and volatilization are not expected (HSDB, 2000).

Metribuzin degradation rate constants are closely related to microbial activity and significantly correlated with the amount of the herbicide available in the soil solution, the Freundlich adsorption coefficient, the clay, sand, and organic matter content of the soil, and the available potassium (Allen and Walker, 1987). The rate of degradation is increased by the activity of soil microorganisms, higher temperatures, and aerobic conditions. The influence of soil microbiological activity on the degradation rate of metribuzin has been demonstrated by several researchers (Bordjiba *et al.*, 2001). Degradation of metribuzinin soil results in different breakdown products (metabolites) including deaminated-metribuzin (DA), Diketo-metribuzin (DK), Deaminated-diketo-metribuzin (DADK) and other metabolites (Pavel *et al.*,

1999). The degradation pathways of metribuzin are shown in Figure



2.

Figure 2.**Metribuzin and proposed degradation pathways(Huertas-Pérez2006)**

(DA – Deaminated-metribuzin; DK - Diketo-metribuzin; DADK - Deaminated-diketo-metribuzin)

This study intends to investigate the presence of soil bacteria adapted for metribuzin degradation in NRDB with the ultimate goal of developing a culture for bioremediation of metribuzin contaminated soils, using bioaugmentation methods.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

Detailed information about the study site can be found in a Kenya government report (GOK, 2014). In summary, Nzoia Sugar Company is in Bungoma County which slopes from the foot of Mt. Elgon. Five major rivers drain the land; rivers Nzoia, Lwakhakha, Kuywa, Chwele and Khalaba. Five major rivers drain the land; rivers Nzoia, Lwakhakha, Kuywa, Chwele and Khalaba. The county has a two-season rain regime, the long rains covering March to May while the short rains start in October to December. Temperature variations are very moderate ranging from 21-25°C during the year. The county is endowed with well-drained, rich and fertile arable soils.

The population of the county statistics of 2009 was 1,374,627 (671,222 males and 703,405 females) (GOK, 2014). It is evenly distributed with an average population density of 482 persons per square km. There are heavier population concentrations in the main urban centres and major factories. These include Nzoia Sugar Company, Malakisi Tobacco Leaf Centre, Bungoma Town, Kimilili, Sirisia, Chwele and Tongaren. Urban population is about 30 per cent of the total population. The main economic activity is subsistence agriculture with maize, sunflower, sugarcane, coffee, tobacco, potatoes and beans being the main crops, and some cattle farming. Agricultural production provides 60 percent of all household incomes in the area.

Nzoia Sugar Company, within which the study farms are found is situated at a latitude of 0˚35'N and a longitude of 34˚40'E, and an altitude of between 1420-1490 meters above sea level. Annual rainfall experienced by the company ranges between 1500-1800mm (GOK, 2014).

Farms with history of metribuzin use were purposively identified. Nzoia Sugar Company has designated seven regions (districts) consisting of subdivisions of farms. Three regions (region 3, 4 and 5) were purposively identified to having farms that had metribuzin lastly used not more than three years from time of sampling. Five farms were identified and simple random soil sampling method (Pleysier, 1995) was used to sample. The farms were identified and coded according to designated numbers and regions used by Nzoia Sugar Company. At the time of sampling the sugarcane in the farms was either ‘Ratoon’ (old crop harvested and allowed to sprout) or ‘Plant crop’ (vegetative stems grown on freshly prepared

land). Field temperatures were taken at the time of sampling. The map of study area is shown in Figure 3.

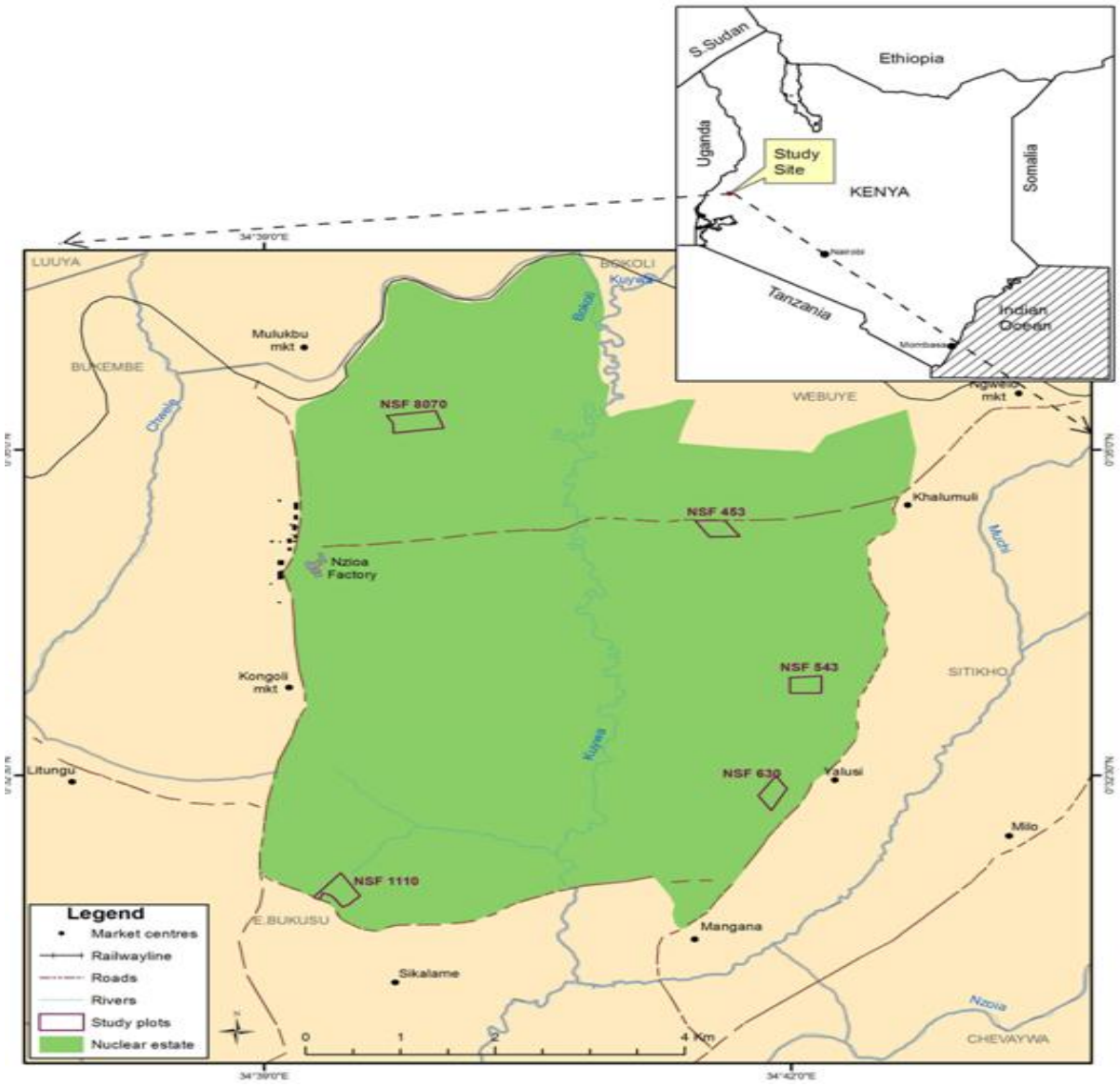


Figure 3: Map of study area with designated study farms, courtesy of G. Maina, Department Environmental Science, Egerton University and Nzoia Sugar Company.

3.2 Soil samples collection and preparation

The soils were obtained from a depth of 0 - 10cm in triplicates from each of the five farms. Soil temperatures at the depth of 10cm were measured in the field using laser thermometer, (Raytek® Model – RAYRPM30L2G, USA). The samples were labelled and put in a cool box for transportation to the laboratory in readiness for the experiments. In the laboratory the soil samples were sieved though 2mm sieves to remove gravel and debris.

3.3 Measurement of soil pH

Soil pH was measured by standard method described by Geotechnical Engineering Bureau (2007) as follows. A homogenous representative composite sample was made and sieved through a 2mm sieve. Thirty grams of soil was weighed and put in a glass beaker. 30mL of distilled water was added to the sample and stirred. The sample was left to stand for one hour, stirring every 10 - 15mins to allow the 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 method described by Black (1965). Aluminium tin was weighed and weight recorded (tare). Ten grams of soil was weighed in the tin and placed overnight in an oven at 105^{o}C temperature. The sample was weighed and weight recorded. The soil sample was returned in the oven, until no difference between any two consecutive measurements was recorded. The formula used to determine moisture content was as follows;

$$\text{Moisture content} = \frac{(\text{wt of wet soil + tare}) - (\text{wt of dry soil - tare})}{(\text{wt of dry soil + tare}) - (\text{tare}).}$$

3.4 Determination of bacteria population

Bacteria viable counts were determined by method described by Curtis *et al.* (2000). Serial dilutions of, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were prepared from soil samples from the five farms using sterile distilled water. Triplicates of 1mL of 10^{-4}dilution were put in sterile Petri dishes and molten nutrient agar was added and thoroughly mixed for bacteria growth using pour plate method. The plates were incubated at a temperature of 35^{o}C and observation of results was done after 48 hours. Bacterial colonies were counted using a colony counter. Viable Colony Forming Units (CFUs) were counted and total numbers per gram of soil determined by multiplying with the dilution factor.

3.5 Isolation of metribuzin degrading bacteria

Isolation of metribuzin degrading bacteria was carried out using selective enrichment method described by Zhang *et al.,* (2009). Portions of ten grams of 0–10 cm composite soil samples were aseptically added to sterile 90 mL of carbon-limited mineral salts medium. The media constituted of1.5 g K_2HPO_4, 0.5 KH_2PO_4, 1.0 g $(NH4)_2SO_4$, 0.5 g NaCl, 0.2 g $MgSO_4$, 0.02 g $FeSO_4$, at pH 7.2. The mixture wasplaced in a 250 ml flaskcontaining 10mgkg^{-1} of analytical grade metribuzin as final concentration. In this medium metribuzin was the sole

carbon source for microbes. Incubation was done at 35ºC in a shaker for seven days. The enriched culture was spread on mineral salts agar culture plates containing 10mg/L metribuzin. Different colonies were picked up, and further purified by streak plate method. Seven pure cultures were isolated and grown on nutrient agar slants and stored in the refrigerator at 4ºC until required in subsequent experiments.

3.5.1 Morphological identification of metribuzin degraders

The isolates were transferred from mineral salts media to growth media containing beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, agar 15.0 g at pH 7.0. The morphological, cultural characteristics and Gram stain reaction of the isolates were determined by microscopy. Gram stain and cell morphology data were recorded.

3.5.2 Biochemical Tests

Biochemical tests that were performed were oxidase reaction; urease and catalase tests, motility test, gelatine liquefaction, starch and casein hydrolysis, and acid production in glucose. These were done as described in Bergys Manual of Systematic Bacteriology (Bergey and Holt, 1994). Results were recorded as positive (+) or negative (-).

3.6 Determination of growth conditions of the isolates

3.6.1 Temperature

The selected isolates were cultivated in growth media containing beef extract 5.0 g, peptone 10.0 g, sodium chloride 5.0 g, pH 7.0 and subjected to incubation temperatures of 20ºC, 27ºC and 35ºC to determine the effect of different temperatures on growth of the isolates. McFarland solution of optical density (OD) 0.2 was prepared (NCCLS, 1990). The solution was used as a guide to prepare bacterial suspensions of 0.2 OD for each isolate as the initial concentration of bacteria cells, before incubation. Incubation was done for 48hrs and optical densities (OD_{600}) were measured using spectrophotometer (Thermofisher scientific, Genesys 10-S model, Germany), to establish best growth temperatures for the isolates and data was analysed by descriptive statistics.

3.6.2. Determination of optimal pH for growth of isolates

The pH of the media was adjusted to 5.0, 7.0 and 9.0. Adjustments were done using dilute hydrochloric acid and dilute sodium bicarbonate. Each isolate was cultivated into the media with adjusted pH and incubated at 35ºC for 48 hrs. The optimal pH for the growth of

the isolates was determined by measuring optical densities (OD_{600}) on the spectrophotometer and recorded.

3.6.3 Determination of effect of Nitrogen concentration on growth of isolates

Calcium ammonium nitrate (analytical grade) was used to adjust nitrogen concentration in the growth media. Concentrations of 5, 7.5 and 10 gL^{-1} were added to growth media. Bacterial cells of OD 0.2 were prepared for each isolate as the start cells concentration before incubation. Each isolate was grown in each nitrogen concentration and optical densities (OD_{600}) of the cells were measured to determine growth performance of the isolates in the different nitrogen levels. The negative control consisted of bacterial cells of OD 0.2 in growth media, incubated for 24hrs, same time as test samples. The difference in growth between the test and negative control was determined as the growth due to nitrogen effect as presented in the result.

3.6.4 Determination of effect of Phosphorous concentration on growth of isolates

Di-ammoniun phosphate analytical grade was used to make phosphorous concentrations of 5, 7.5 and 10 g L^{-1}in the growth media. Bacterial cells of OD 0.2 were prepared for each isolate as the start cells concentration before incubation. Each isolate was grown in each phosphorous concentration and optical densities (OD_{600}) of the cells were measured to determine growth performance of the isolates in the different phosphorous levels. The negative control consisted of bacterial cells of OD 0.2 in growth media, incubated for 24hrs. The difference in growth between the test and negative control was determined as the growth due to phosphorous effect as presented in the results.

3. 7 Determination of metribuzin degradation potential of the isolates

Seven metribuzin degrading isolates were grown at 35^{o}C growth temperature for 24 hours in nutrient broth. They were inoculated subsequently in mineral salts media containing 10$mgL^{-1}$of metribuzin at an $OD_{600}$of ≈ 0.2 optical density. The cultures were incubated in triplicates and optical densities taken at various time intervals until optical densities remained constant. This was taken as the end point of metribuzin degradation by the isolates and corresponded to an incubation period of 21 days. Results were recorded in a graph showing growth of the isolates (OD_{600}) versus time in days. The amount of metribuzin degraded was determined using high performance liquid chromatography (HPLC) by method described by Papadakis and Papadopoulou (2002) as follows. After optical densities were constant; between day 12 and day 21 of incubation, samples were filtered through 0.2µm filters prior to

HPLC quantification of the pesticide residues. Standards of metribuzin and metribuzin metabolites namely; DA, DK, DADK were preparedin concentrations of between 2.05mgL^{-1} and 21.51 mg$L^{-1,}$ in consideration of the initial test metribuzin concentration at 10 mgL^{-1} and the capacity of the HPLC.'Shimadzu Prominence' HPLC system model LC-20AT was equippedwith prominenceUV/Vis, SPD-20AVdetector and auto sampler, SIL-20A HT and a C-18 column (250 mm ×4 mm). The column was operated at ambient temperature with a flow rate of 0.8 mL min^{-1} and an injection volume of 20µL. An isocratic mobile phase system was established using acetonitrile: water at a ratio of 60: 40. This ratio enabled the separation and quantification of metribuzin in a single HPLC run of 8 min at 293 nm. The retention time was 3.2 minutes. Standard curves were constructed for metribuzin and the metabolites and r-squared values for each curve determined. Residual amount of metribuzin and metabolites for different isolates were determined at the end of degradation period, presumed to be when optical densities of isolates remained constant with time. The same aliquots for optical densities readings were used to determine the residual metribuzin and metabolites and amounts estimated from the standard curves for each metabolite.

3.8 Molecular characterization and identification

The identification of isolates was based on colony morphology, cultural and biochemical characteristics, following *Bergey's Manual of Systematic Bacteriology* (Holt *et al.,* 1994).DNA was extracted using the phenol-chloroform method on seven isolates (NZ453A, NZ453B, NZ453C, NZ543A, NZ543B, NZ8070 and NZ1110) that showed ability to degrade metribuzin. The 16SrRNA gene was amplified by using polymerase chain reaction (PCR) with the universal primer pair (Hang *et al*., 2003) of 16SF5'AGAGTTTGATCCTGGCTCAG 3' and 16SR5'GTACGCTACCTTGTTACGAC 3' The conditions for PCR were: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, at 48°C for 1 min, and 72°C for 3 min, and a final extension at 72°C for 10 min. PCR fragments were purified by the Gene Jet™ extraction Kit.

The 1445 base pairs of 16S rRNA gene sequence were determined at International Livestock Research Institute (ILRI), Kenya. Sequences were extracted from the database and aligned. Software DNAMAN 4.0 (LynnonBio-Soft, Vaudreuil, Canada) and CLASTAL X 1.83 (Thompson *et al*., 1997) were used for sequence analysis and contrast. The Sequences were compared against available DNA sequences in GenBank.

3.9 Research design

The study was carried out using experimental research design. Soil samples obtained from the farms were taken to the laboratory, for isolation of unknown potential metribuzin degrading bacteria. Positive and negative controls were prepared in the laboratory as standards in the experiment.

3.10 Data analysis

Data was presented in tables and graphs generated using Microsoft office version 2007 program suite. Statistical analysis on the data was performed using SPSS program version 9. Descriptive statistics was used to analyse distribution of bacteria in the farms and compare the number of metribuzin degrading isolates from different farms. Analysis of variance (ANOVA) was used to test significant differences in the effects of temperature, pH, and inorganic nutrient concentrations on the growth of metribuzin degrading bacteria. Means of growth variables that were significantly different for each isolate were determined by separation of means using LSD. Regression analysis was used to analyse levels of degradation of metribuzin by the different isolates. Significant results were determined at probability $p < 0.05$. Summary of methods of data analysis are shown on Table 1.

Table 1: Summary of methods of data analysis

Objective	Method of analysis
To determine bacteria population distribution, isolate and characterize bacteria with potential of degrading metribuzin in Nzoia sugar company farms.	Descriptive statistics, separation of means by LSD, pearsons' correlation statistics.
To determine influence of temperature, pH, phosphorous and Nitrogen on growth of metribuzin degrading isolates.	Descriptive statistics, separation of means by LSD.
To determine degradation potential and molecular identities of metribuzin degrading bacteria	Descriptive statistics, regression analysis

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1. Determination of bacteria population distribution

Soils of study farms, coded NSF (Nzoia Sugar Farm) had total viable bacteria counts as shown in Table 2. Distribution population of total bacteria counts among the farms is presented in Figure 4.

4.1.1. Distribution of total viable bacteria counts among the farms

NSF680 recorded the lowest, numbers of total viable bacteria while the highest was recorded on NSF1110 as indicated in Table 2. Statistical analysis showed significant difference at $p < 0.05$ in viable counts in all farms except farms for NSF453 and NSF680.

Table 2: Mean bacterial numbers (CFUs, $\times 10^5$ per gram of soil) among the farms.

Farm code	Mean ± SD
NSF 453	6.3 ± 0.187^a
NSF 543	7.5 ± 0.199^b
NSF 680	6.1 ± 0.040^a
NSF1110	18.7 ± 0.068^c
NSF8070	13.5 ± 0.092^d

Means with the same letter are not significantly different, **P<0.05**

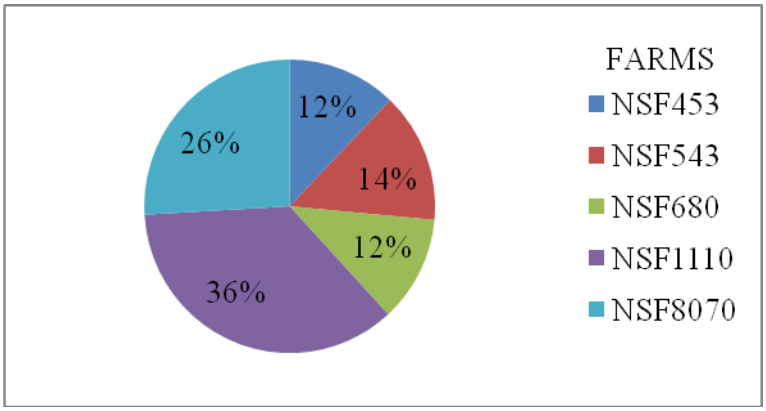


Figure 4: Chart showing percentage distribution of total bacteria counts among farms.

Differences in numbers, biomass and diversity of soil microorganisms, their distribution and activities depend on physical-chemical characteristics, nutrients, presence of

interacting organisms and other activities carried out in the soils environment, (Hossain, 2012). Bacteria in natural environment have a cluster distribution due to availability of physical and chemical resources at microhabitat and micro niche levels in the environment (Hossain, 2012). Differences in distribution of total viable bacteria and metribuzin degrading bacteria, among farms of Nzoia Sugar Company could have been influenced by the different abiotic factors in the farms. The different farms could have developed different abiotic parameters due to different agricultural activities carried out in the farms among other factors.

4.1.2 Influence of soil physico-chemical parameters on total bacteria population

Table 3 show means and standard deviations of pH, field temperatures soil moisture content and viable bacteria counts of the study farms soils. Farms were coded as Nzoia Sugar Farms (NSF) followed by a designated number that is used by Nzoia Sugar Company to identify the Farms.

Table 3: Physico-chemical characteristics of study farm soils

Farm Code	Soil pH Means ±SD	Field soil temperature (^{o} C) ± SD	Soil moisture content (%)±SD
NSF8070	5.00 ± 0.28	16.00 ± 2.00	16.00 ± 2.00
NSF453	4.80 ± 0.70	23.00 ± 1.53	17.00 ± 0.58
NSF680	4.60 ± 0.09	28.00 ± 0.58	21.00 ± 1.00
NSF543	4.60 ± 0.15	25.00 ± 0.58	21.00 ± 2.08
NSF1110	5.50± 0.28	28.00 ± 0.58	16.00 ± 3.21

Soil moisture content varied moderately between 16% (NSF8070 and NSF1110) and 21% (NSF 680 and NSF543). Soil pH did not vary largely among the farms studied. The highest pH value (5.5) was found in NSF1110 and the lowest (4.6) was found in NSF680 and NSF 453. The highest values of field soil temperatures were 28^{o}C (NSF680 and NSF1110 and lowest was 16^{o}C (NSF8070).

Correlation results between soil physico-chemical parameters and bacteria population counts are shown in Table 4. Results indicated that number of viable bacterial counts was significantly and positively correlated with only pH rather than soil temperature and soil moisture content. This indicates the importance of the soil pH in driving the bacterial population in the study area. The result of the present study correspond with the findings of other studies (Ezeigbo *et al.*, 2013) that reported that pH was more important than moisture

and soil temperature for bacteria in agricultural farms. This was attributed to pH directly affecting the solubility of elements, of which essential minerals can become unavailable, at extremes of pH (> 2 and < 11) (Rothschild, 2002).

Table 4. Correlation coefficients (r) of the physico-chemical properties with the total viable bacterial counts ($CFUg^{-1}$ soil) in the soils of Nzoia sugar company (n = 5)

Soil physical chemical properties	r
Moisture %	-0.446^{ns}
pH	0.549*
Temperature	-0.040^{ns}

* and ns indicate significant and not significant respectively.

A study by Rousk *et al.* (2009) on effect of soil pH on microbial biomass observed that pH influences bacteria biomass, but noted it was not pH itself that was responsible for changes in bacterial biomass; but that pH gradients can decrease or increase availability of nutrients for microorganisms, resulting in decrease or increase in bacterial biomass respectively.

4.1.3. Morphological and biochemical characterization of the bacteria isolates

Seven metribuzin degrading bacteria were isolated from the farms. The numbers were isolated from the farms as follows; NSF453 had three metribuzin degrading isolates, the highest number, followed by NSF543 with two isolates; NSF1110 and NSF8070 had one isolate each. None was isolated from NSF680. The bacteria isolated from the study farms were coded 'NZ' for Nzoia, followed by the farm number. Their characteristics were determined by cultural, cell morphology and biochemical tests as shown in tables 5 and 6, respectively. Photomicrographs of the bacteria cultures on growth medium are shown on Plate 1.

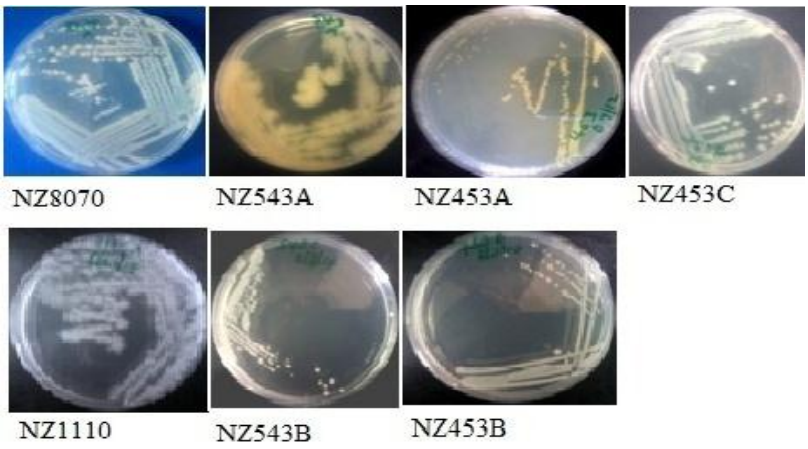


Plate 1: Photomicrographs of metribuzin degrading isolates on growth media.

4.1.4 Cultural, cell morphology and Gram stain reactions of the isolates

Bacterial cultural characteristics portrayed circular, rhizoid and root-like shapes. Five of the metribuzin degrading bacteria isolated, were gram positive while two were gram negative. Five were rod shaped while two were cocci as shown in Table 5.

Table 5: Summary of Cultural, cell morphology and Gram stain reactions of the isolates.

Isolate	Cultural characteristics	Gram reaction	Cell morphology
NZ453B	Circular-white fast growing	Negative	Rods
NZ453C	Circular white colonies	Negative	Rods
NZ453A	Circular-pinpoint - size yellow colonies	Positive	Cocci
NZ543A	Rhizoid white colonies	Positive	Rods
NZ543B	Circular - white - mucoid colonies	Positive	Rods
NZ1110	Root-like spreading white colonies	Positive	Rods
NZ8070	Circular white colonies	Positive	Cocci

4.1.5 Biochemical tests

Biochemical tests carried on the isolates are shown in Table 6. All the isolates were positive for catalyse reaction, but there was a general mixed reaction on other biochemical tests carried out.

Table 6: **Biochemical characteristics of metribuzin degrading bacterial isolates from Nzoia Sugar Company farm**

Bacteria code	Biochemical tests							
	Motility test	Starch hydrolysis	Oxidase reaction	Gelatin liquefaction	Casein hydrolysis	Acid production from glucose	Catalyse reaction	Urease
NZ8070	+	+	-	+	+	-	+	-
NZ453C	+	-	+	-	+	-	+	+
NZ543A	-	+	-	+	+	+	+	-
NZ453A	-	-	+	-	-	+	+	+
NZ1110	+	+	+	+	+	+	+	-
NZ543B	+	-	+	+	+	-	+	-
NZ453B	+	+	+	+	+	-	+	-

The metribuzin degrading isolates portrayed differences in morphological and biochemical characteristics. These differences portray diversity in metribuzin degrading bacteria in Nzoia Sugarcane farms. Diverse degraders are important since they supplement each other's degrading activities and play different roles. In soils, one likely scenario for the complete degradation of herbicides may be the initial enzymatic attack by relatively nonspecific oxidases produced by some microorganisms, followed by further metabolism by hydrolyses and/or ring cleavage enzymes eventually resulting in products which are mineralized via catabolic pathways (Talaro and Talaro, 2002; Ezeigbo *et al.,* 2013). Similar isolation of diverse herbicide degrading bacteria, with different cultural, morphological and biochemical characteristics had also been recorded in other studies (Tamer and Medhat, 2013). Both Gram negative and Gram positive bacteria have been isolated in pesticide contaminated soils in other studies (Sabourmoghaddam *et al*., 2014; Zhang *et al*., 2014).

4.2. Influence of temperature, pH, Phosphorous and Nitrogen on Bacteria growth

Results on growth of isolates at different temperatures, pH levels, Nitrogen and Phosphorous concentrations are shown in Figures 5,6,7and 8, respectively.

4.2.1.Temperature treatment

The growth responses of the isolates are represented in Figure 5. The difference in growth between isolates is presented in Table 7.All isolates showed an increase of growth from 0.2 OD at incubation in all temperatures. Generally NZ543A and NZ1110 showed higher growth in all temperatures of incubation. At 20^{o}C, NZ1110 showed highest growth recorded as OD value of 0.80±0.02. Although there was significant difference in growth among some isolates (Table 7), most of the isolates apart from NZ543C had highest growth at 35^{o}C, (Figure 5).

Metribuzin-degrading soil bacteria from Nzoia Sugarcane farms portrayed different growth temperatures. This has been confirmed in the study, since 5 out of the 7 isolates recorded relatively highest growth at 35^{o}C and the other two had highest growth at 27^{o}C and 20^{o}C each. This indicates capability of the isolates to degrade metribuzin at different temperatures as the weather fluctuates in Nzoia cane farms. Pettersson and Baath (2003) found out that soil bacteria community was better adapted to higher temperatures above 30^{o}C and is confirmed in the study.

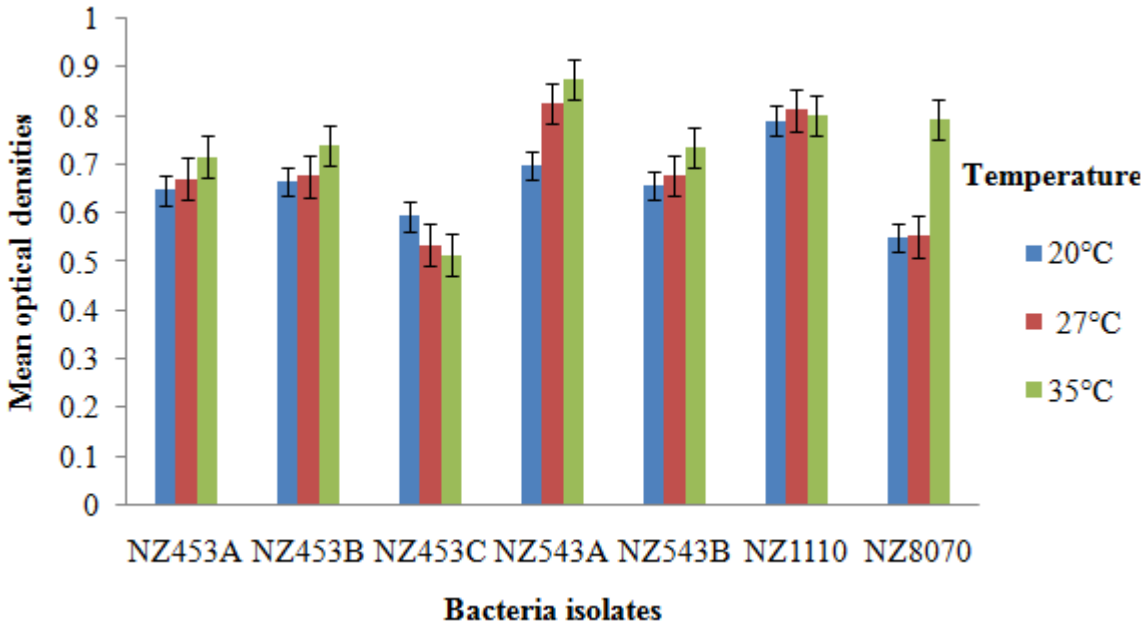


Figure 5: Column Charts showing growth of isolates on different temperatures

Table 7: Difference in growth (OD_{600}) between isolates at different temperatures using separation of means by LSD

	Isolates						
Temperature	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
20° C	0.647^{a}	0.665^{a}	0.593^{b}	0.700^{a}	0.659^{a}	0.791^{c}	0.549^{b}
27° C	0.671^{a}	0.676^{a}	0.535^{b}	0.826^{c}	0.678^{a}	0.812^{c}	0.553^{b}
35° C	0.717^{a}	0.739^{a}	0.514^{b}	0.875^{c}	0.736^{a}	0.801^{d}	0.793^{d}

Same letters denote no significant difference at $p \leq 0.05$

Tamilselvan *et al,* (2014) assessed growth of pesticide degrading bacteria in different temperatures and found the highest growth at 35°C, followed by 25°C and at 20°C. These results were similar with the current study where the highest growth was at 35°C, followed by 27°C and at 20°C.

4.2.2 Growth at different pH levels

The effect of pH on growth of the isolates at different pH levels is shown in Figure 6. pH 9 recorded the highest growth in five of the seven isolates. NZ543A had highest growth of all at pH 7 with OD of 0.89 ± 0.21 while it had the lowest growth of all at pH 5 with OD of 0.38 ± 0.18. Generally, pH 9 reflected the highest pH at which maximum enzymatic reaction

took place in all isolates. At neutral pH of 7, NZ543A recorded relatively, the highest growth. Difference in growth between the isolates is shown in Table 8.

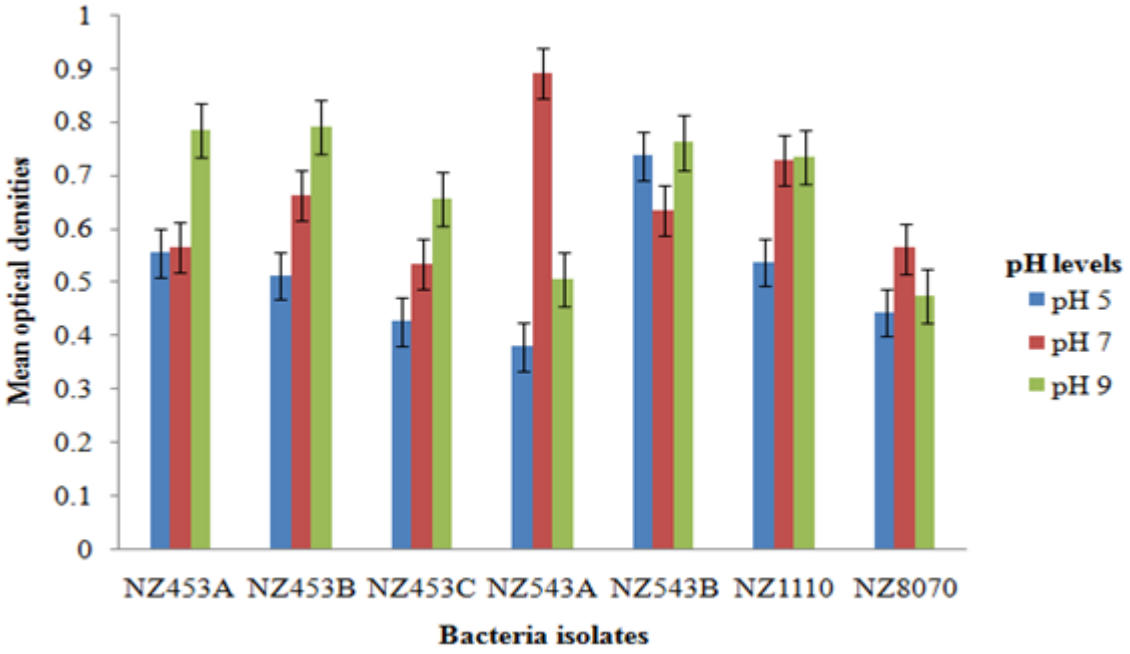


Figure 6: Column Charts showing growth of isolates at different pH levels in growth media.

Table 8: Difference in growth (OD_{600}) between isolates at different pH levels using separation of means by LSD.

	Isolates						
pH	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
pH 5	0.554^{a}	0.511^{b}	0.425^{c}	0.378^{d}	0.786^{e}	0.536^{a}	0.443^{c}
pH 7	0.564^{a}	0.661^{b}	0.534^{a}	0.890^{c}	0.633^{b}	0.728^{d}	0.563^{a}
pH 9	0.784^{a}	0.791^{b}	0.655^{c}	0.506^{d}	0.761^{a}	0.734^{e}	0.473^{d}

Same letters denote no significant difference at $p \leq 0.05$

Generally, the isolates showed significant difference in growth between them at the studied pH. The influence of pH on bacterial growth has been investigated previously. Kamble *et al.* (2014) reported increase of bacteria biomass in twelve soils with increased pH and salinity. Similarly, a study that included 19 different soils from areas with various land uses, spanning a pH range from 4 to 8, showed that there was an increase in bacterial growth with higher pHs as measured by Leucine incorporation (Baath, 1998). Bacterial growth increased fourfold between pH 4 and 8.

Todar (2008) observed that for most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing [H^+] on the rates of enzymatic reaction. Brock *et al.* (2006) gave most bacteria growth range as neutral pH values of between 5 and 8, although some species can grow at more acidic or alkaline extremes.

Tamilselvan *et al.* (2014) assessed growth rate of some pesticide degrading bacteria in different pH media and found out that maximum growth recorded at pH 6, followed by pH 7, pH 8, and pH 5. The lowest growth rate was at pH 4. This differs from current study where the highest growth was at pH 9, followed by pH 7 and then 5. Similarity, both studies had the bacteria having lowest growth as pH becomes more acidic.

4.2.3. Growth of isolates in different nitrogen concentrations

Growth response of isolates in different nitrogen concentrations is shown in Figure 7. Results of difference in growth between isolates are shown in Table 9. Generally, increased growth was observed in concentrations of 7.5gL^{-1} and 10gL^{-1}in different isolates. Highest growth was observed in isolate NZ543A at concentrations of 7.5gL^{-1} with mean OD of 0.88 ±0.21. Isolate NZ543A had the lowest of all growths in nitrogen concentration of 10gL^{-1} with OD of 0.23 ± 0.21.

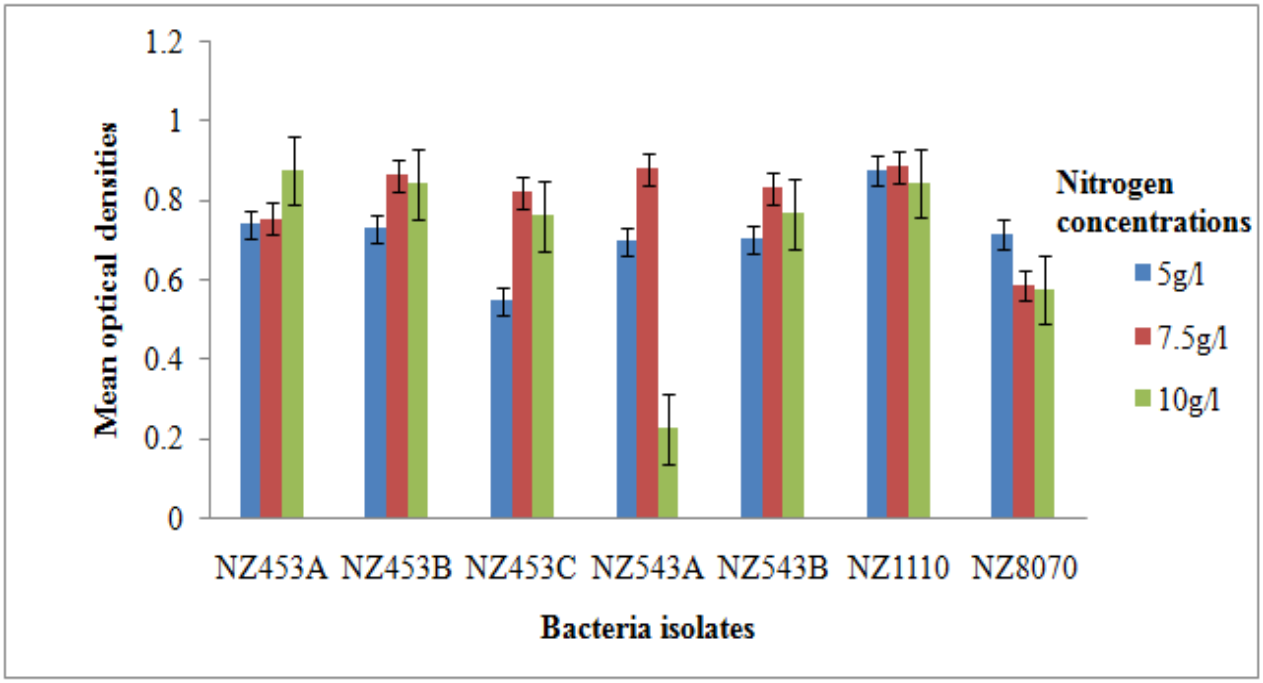


Figure 7: Column Charts showing growth of isolates at different nitrogen concentrations on growth medium.

Table 9: Difference in growth (OD_{600}) between isolates at different nitrogen concentrations using separation of means by LSD

	Isolates						
Nitrogen	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
5gL^{-1}	0.739^{a}	0.728^{a}	0.546^{b}	0.695^{a}	0.701^{a}	0.874^{c}	0.713^{a}
7.5gL^{-1}	0.753^{a}	0.860^{b}	0.82^{c}	0.876^{b}	0.830^{c}	0.883^{d}	0.586^{e}
10gL^{-1}	0.875^{a}	0.841^{b}	0.76^{c}	0.225^{d}	0.765^{c}	0.843^{b}	0.574^{e}

Same letters denote no significant difference at $p \leq 0.05$

Addition of nitrogen into growth media showed increased growth in all bacteria isolates though growth intensity varied among isolates. Cleveland *et al.* (2002) observed that additions of nitrogen to nitrogen -poor systems did not result in consistent effects upon decomposer communities. In some cases, nitrogen did appear to stimulate microbial growth, but in many others its addition had no effect, or sometimes even a negative effect. The study was based on addition of nitrogen in soils and subsequent count of bacterial community biomass. Increase in growth in the current study could be due to adequate availability of other nutrients including carbon in the media with nitrogen further enriching the medium.

4.2.4. Growth in phosphorous concentrations.

Figure 8 shows response in growth of the isolates in different concentrations of phosphorous. The highest growth was in NZ1110 at concentrations of 10gL^{-1} with OD of 0.93 ± 0.04 while the lowest was in NZ543A with OD values of 0.21 ± 0.04, at 10 gL^{-1}phosphorous concentration. Difference in growth between isolates is shown in Table 10.

There was a general difference in growth between isolates as reported in Table 10. The isolates under study showed higher growth in high concentrations of inorganic phosphorous. Out of the seven isolated soil bacteria, five had highest growth in phosphorous concentration of 10 g L^{-1} and two had highest growth at phosphorus concentration of 7.5 gL^{-1}.

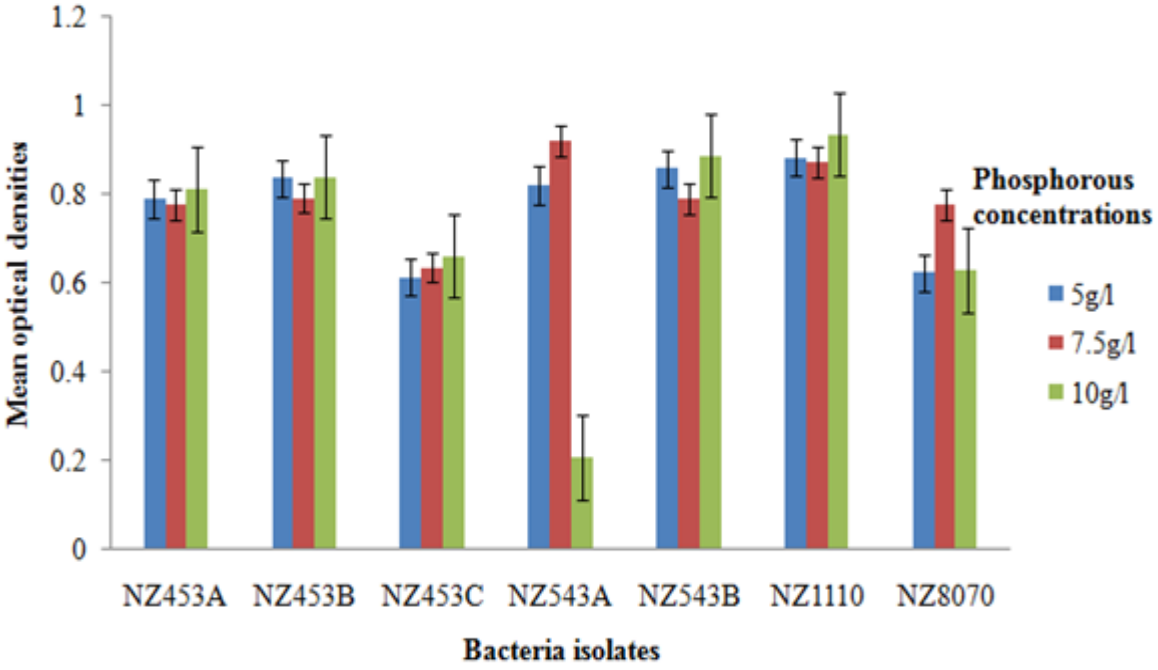


Figure 8: Column Charts showing growth of isolates at different phosphorous concentrations in growth medium.

Table 10: Difference in growth (OD_{600}) between isolates at differentphosphorous concentrations using separation of means by LSD.

	Isolates						
Phosphorous	**NZ453A**	**NZ453B**	**NZ453C**	**NZ543A**	**NZ543B**	**NZ1110**	**NZ8070**
5 gL^{-1}	0.791^{a}	0.837^{a}	0.614^{b}	0.821^{a}	0.860^{c}	0.884^{c}	0.624^{b}
7.5 gL^{-1}	0.778^{a}	0.793^{a}	0.637^{b}	0.921^{c}	0.791^{d}	0.873^{d}	0.778^{a}
10 gL^{-1}	0.812^{a}	0.841^{b}	0.661^{c}	0.208^{d}	0.889^{b}	0.936^{e}	0.630^{c}

Same letters denote no significant difference at $p \leq 0.05$

Phosphorus has a major ecological role in nature, because it is an essential element for microbes and because it is commonly the least abundant element compared to carbon (Cleveland and Liptizin, 2007). Griffiths *et al.* (2012) observed that addition of phosphorus fertilizer in soil led to saturation of microbes unlike when carbon and nitrogen were added alone. Increase in bacteria growth was also observed in the current study, with addition of phosphorous in nutrient broth. In bio-stimulation studies nutrients such as phosphorus and nitrogen are required to speed up biodegradation of pollutants (Adams *et al.,* 2015). This means that when nitrogen and phosphorous based fertilizers are added in the farms, there is a

possibility that bacteria would enhance degradation of residual pesticides that may exist in the soil.

4.2.5 Summary of most favourable of the selected growth conditions of the isolates

The optimal growth conditions of isolates in different concentrations of phosphorous, nitrogen, at different pH and temperature as determined by highest optical densities (OD_{600}) are summarized in Table 11. Five isolates showed optimal growth conditions in temperature of 35 º C; while one each had optimal growth at 20 and 27 º C. Five of the isolates had optimal growth at pH 9, two isolates at pH 7. In phosphorous, five isolates had optimal growth at a concentration of 10mg/L, while two at a concentration of 7.5mg/L. None had optimal growth at $5gL^{-1}$. In nitrogen, three isolates had optimum growth in concentrations of 10 gL^{-1} and 7.5 gL^{-1}. Only one isolate showed optimal growth in 5 gL^{-1} of nitrogen.

Table 11: Summarized data showing bacteria isolates and physico-chemical parameters that recorded the highest growth for each isolate

Isolate	Temp º C	pH	Phosphorous gL^{-1}	Nitrogen gL^{-1}
NZ453C	35	9	10	10
NZ453B	35	9	10	7.5
NZ453A	20	9	10	7.5
NZ543A	35	7	7.5	7.5
NZ543B	35	9	10	10
NZ1110	27	9	10	10
NZ8070	35	7	7.5	5

4.3 Determination of degradation potential and molecular identities of the bacteria isolates

Standard curves generated in determination of isolates degradation potential for metribuzin and subsequent metabolite standard curves are presented in appendices, i, ii ,iii and iv. Metribuzin degradation profiles are presented in Fig. 9. Bacteria cells of OD_{600} 0.2 for each isolate was put into the media as the the initial cell concentration. Duration of metribuzindegradation ranged fromzero to 21st day but slowed down from day 12. Degradation end point wasdetermined when optical density values of growth of isolates in Mineral Salts Media remained constant. Isolate NZ543A presented a relatively fast,

shortlived and highest Optical densities over time compared to the rest of the isolates. NZ453B and NZ543B presented, relatively the longest time of degradation.

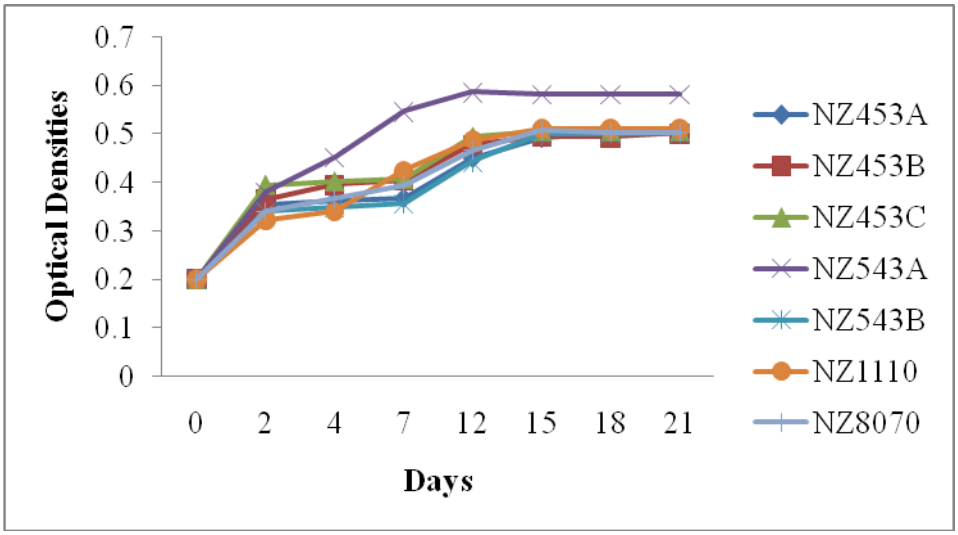


Figure 9: Line graph showing Growth(OD) trends of the isolates in MSM against time (Days).

The general trend of growth of the isolates in MSM showed an increase in growth between day zero and day 2. This could be due to traces of nutrients transferred from growth media used to multiply the cells into MSM, the test medium. Traces of these nutrients were easier to breakdown than nutrients in MSM. Generally, growth slowed down between day 2 and day 7. This could be due to the isolates adapting to grow in the Mineral salts medium, and after adapting growth increased, and ceased between day 12 and 21.

4.3.1 Determination of residual metribuzin

Average peaks of residual metribuzin obtained on HPLC runs, with calculated concentration of residual metribuzin in $mgkg^{-1}$is shown in Table 12. Isolate NZ543A had undetectable levels of metribuzin with the method used. NZ453A had the highest residual metribuzin.

Table 12: Residual metribuzin in mg kg^{-1} after degradation by the isolates

Bacteria	Conc (mg Kg^{-1})
NZ453B	0.3764
NZ453C	0.3488
NZ453A	0.7417
NZ543A	Not Detected
NZ543B	0.4682
NZ1110	0.2666
NZ8070	0.3098

4.3.2 Determination of degradation capability of the isolates

Degradation levels determined by the difference between the initial concentrations of metribuzin of 10mg$Kg^{-1}$and the residual metribuzin obtained after HPLC procedure, ranged between 9.3 to 9.73 mgKg^{-1} as shown in Table 13. The difference was regarded as the degradation potential of each isolate and was expressed in percentage. The bacterial isolates degraded between 93% and 97.3% of metribuzin.

Table 13: Bacteria isolates' degradation potential calculation

Initial metribuzin conc. MgKg^{-1} (a)	Isolate	Residual metribuzin MgKg^{-1} (b)	Degraded metribuzin MgKg^{-1} (a-b)	% degradation
10 mgKg^{-1}	NZ453B	0.38	9.62	96.2
	NZ453C	0.35	9.65	96.5
	NZ453A	0.7	9.3	93
	NZ543A	Not Detected		
	NZ543B	0.46	9.54	95.4
	NZ1110	0.27	9.73	97.3
	NZ8070	0.31	9.69	96.9

Previous studies carried out elsewhere have portrayed several bacteria with high potential to degrade several xenobiotics compounds. A study by Vasques and Reyes (2002) evaluated three *pseudomonas* species for degradation of herbicide aroclor1242 and reported that these bacteria had a great ability of degrading the herbicide with a percentage of; 99.8,

89.4 and 98.4 respectively. These high degradation potential of the bacteria were observed in the current study, though with a different herbicide.

A study done by Zhang *et al* (2014) reported isolation of a *Bacillus sp* which degraded 73% of 20mg L^{-1} of metribuzin and 45% of 100mgL^{-1} after 120 hrs in soil. This can be regarded as high rate of degradation in a shorter time than was in the case of current study where by degradation was measured after 21 days. The degradation study was carried out in soils, unlike in the current study where experiments were *invitro*. The use of controlled microbial growth conditions and use of individual bacteria isolates in degradation tests in current study could have contributed to the difference in degradation results obtained in both studies. High degradation rates after a shorter period of time as reported by Zhang *et al.* (2014) could be due to co-metabolism of metribuzin by a combination of microbes in the soil unlike in the current study where individual pure culture bacteria isolates were used.

4.3.3 Determination ofmetabolitesformed inmetribuzin degradation

Metribuzin in soil is degraded through deaminated-metribuzin (DA), Diketo-metribuzin (DK), Deaminated-diketo-metribuzin (DADK) and other metabolites (Pavel *et al.*, 1999). Presence of these metabolites was an indication that metribuzin was degraded by the bacterial isolates through that pathway. The concentrations of these metabolites are presented in Table 14.

Table 14: Metabolite concentrations recovered after degradation of metribuzin by isolates.

Bacteria	DK	DADK	DA (mgkg^{-1})
NZ453B	0.7270	0.1732	0.2691
NZ453C	0.9398	0.0917	0.4758
NZ453A	0.3684	0.3127	0.0463
NZ543A	0.7344	0.0315	0.3425
NZ543B	Not detected	0.5525	0.0369
NZ1110	Not detected	0.1153	0.4492
NZ8070	Not detected	0.3942	Not Detected

Some of the metabolites were not detectable through the method used. This could be attributed to production of other metabolites as Pavel *et al.,* (1999) cited that, other than DA, DK and DADK, other metabolites can be produced by metribuzin degradation. Metabolite DA is formed through the deamination of metribuzin (Khoury *et al*., 2006). Metabolite

DADK is a product of reductive deamination of metribuzin. Metribuzin undergoes two different pathways to yield DADK. One route involves, deamination of metribuzin to form DA which then undegoes oxidative desulfuration to produce DADK. The other pathway involves oxidative desulfuration of metribuzin yielding diketo-metribuzin (DK) which then undergoes deamination (Henriksen *et al*., 2002). Therefore, isolates NZ543B, NZ1110 and NZ8070 may have favoured the deamination of metribuzin to form DA which then underwent oxidative desulfuration to produce DADK. Else, if DK was produced by these isolates, it may have been below detection limits due to its transformation toDADK through deamination. Total degradation of metribuzin is reported to occur through DADK (Henriksen *et al*., 2002; Khoury *et al*., 2003).Thus the detection of DADK implies possible complete mineralization of metribuzin in these soils by identified bacterial isolates.

4.3.4 Molecular Characterization

4.4 Polymerase chain reaction

The seven metribuzin degrading isolates were amplified and identified by 16S rRNA sequence analysis. Figure 10 shows the Gel electrophoresis picture of 16S rRNA amplifications.

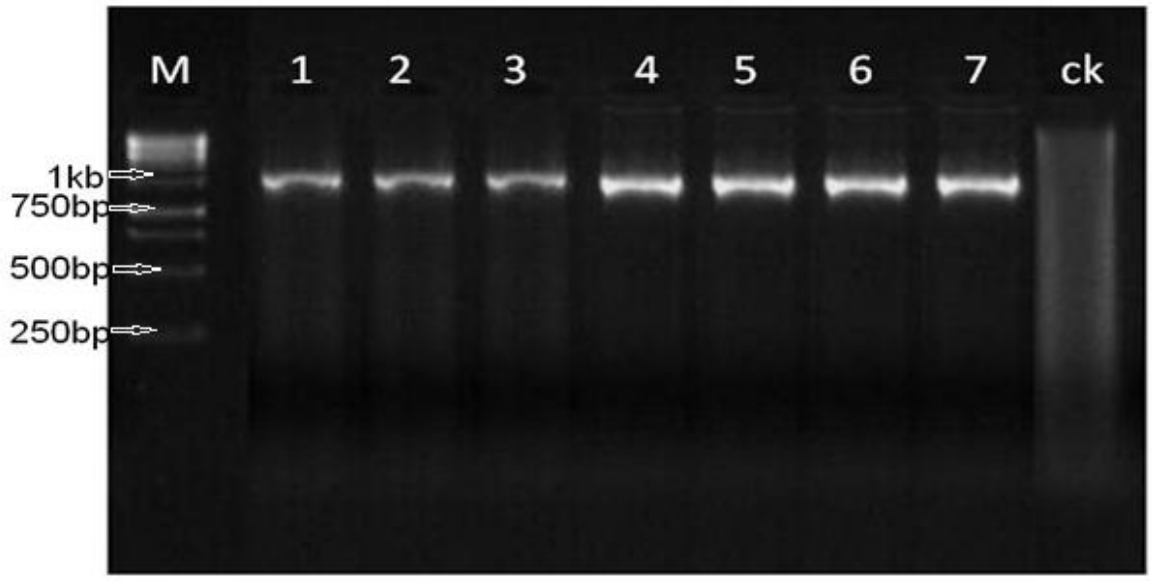


Figure 10.PCR products of 16SrRNA gene amplification. M**: marker (1Kb ladder),** 1-7**DNA of isolates and ck is the control.**

4.4.1 Phylogenetic analysis

The similarities of the isolates to other bacteria in the gene bank are illustrated in phylogenetic trees in Fig.11, 12, 13, 14, 15, 16 and 17. Phylogenetic relationship of NZ453A (*Staphylococcus sp.)* NZ543A and NZ1110 *(Bacillus sp.),* NZ453 C *(Pseudomonas sp.),*

NZ8070 (Planococcus sp.), NZ435B *(Burkholderia sp.), and NZ543B (Arthrobacter sp*.) isolated from the soil in Nzoia sugarcane farms Kenya compared with other isolates of *Bacillus sp, Pseudomonas sp., Planococcus sp., Burkholderia sp., Staphylococcus sp., and Arthrobacter sp*. based on complete and partial nucleotide sequences available in the GeneBank. The sequences were aligned and trees constructed using Mega 6.06 package neighbour joining and bootstrap value of 1000 replicates.

BLAST analysis revealed that isolates were similar to previously identified bacteria.NZ453C showed 97% similarity to *pseudomonas putida*, NZ8070, had 97%, similarity to *Planomicrobium flavidum*, NZ1110 was 98% similar to *Bacillus amyloliquefaciens*, NZ453B, 98% to *Burkholderia cepacia*, NZ543B, 98% to *Arthrobacter globiformis*, NZ453A had 97% similarity to *staphylococcus sciuri* and NZ543A 99% to *Bacillus pseudomycoides*. Bacteria isolates were assigned accession numbers from gene bank as shown in Table 15.

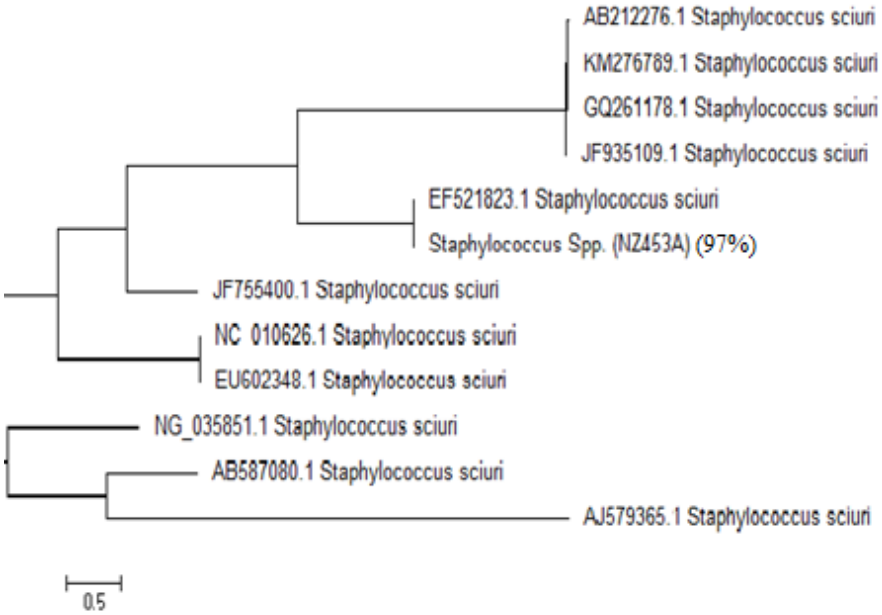


Figure 11. Phylogenetic tree of Isolate NZ453A

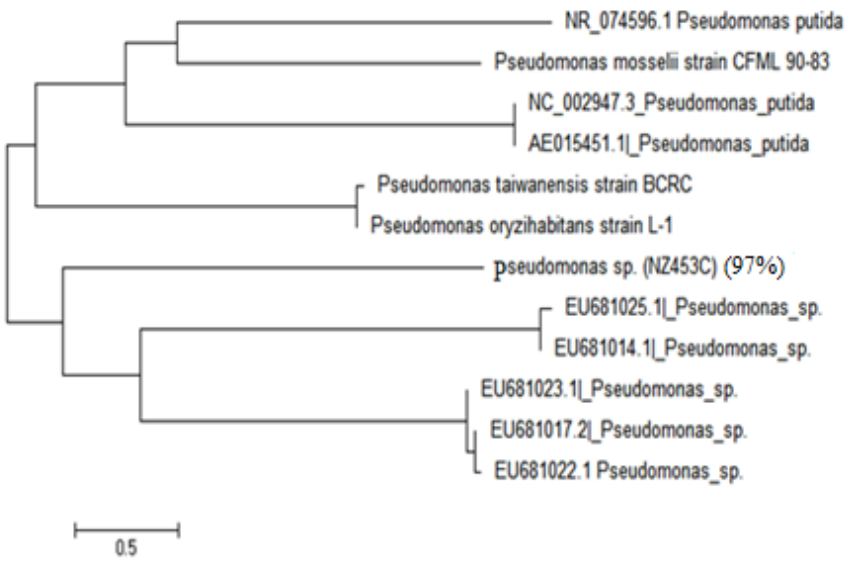


Figure 12. Phylogenetic tree of isolate NZ453 C

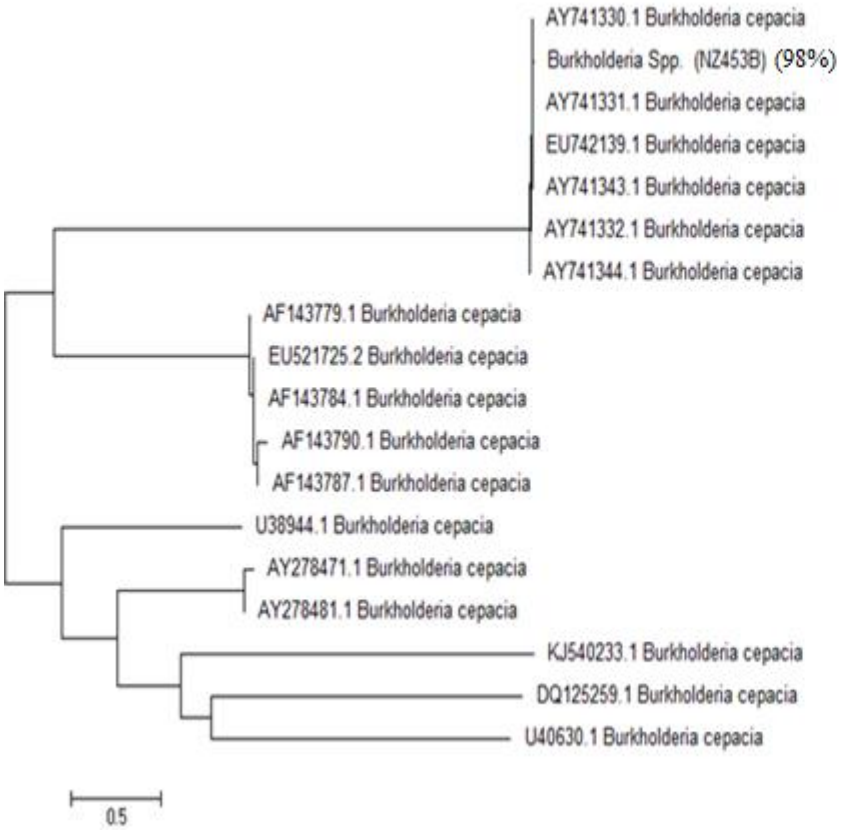


Figure 13: Phylogenetic tree of isolate NZ435B

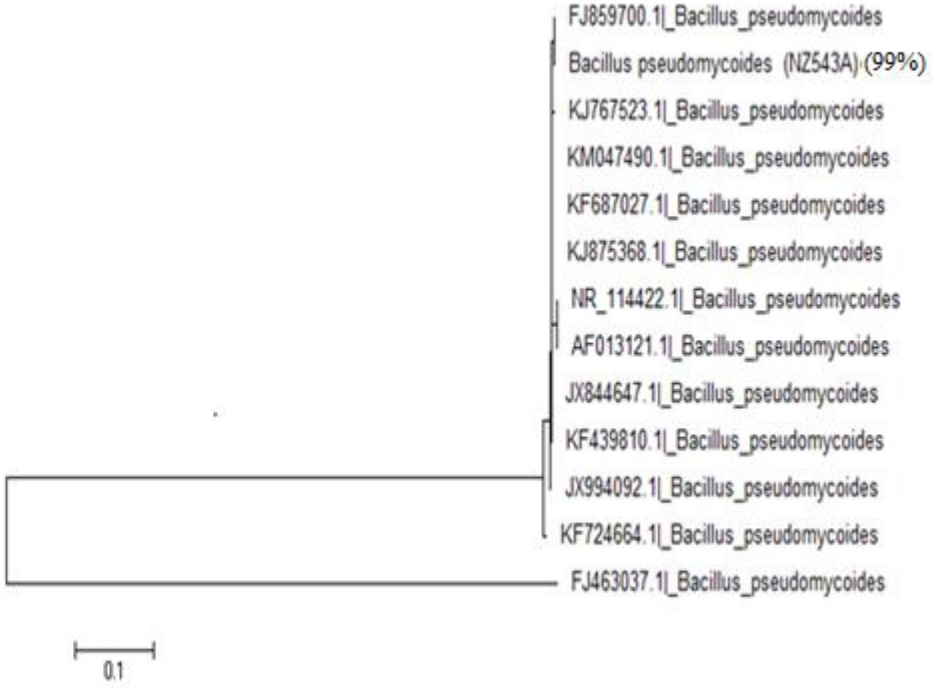


Figure 14: Phylogenetic tree of isolate NZ543A

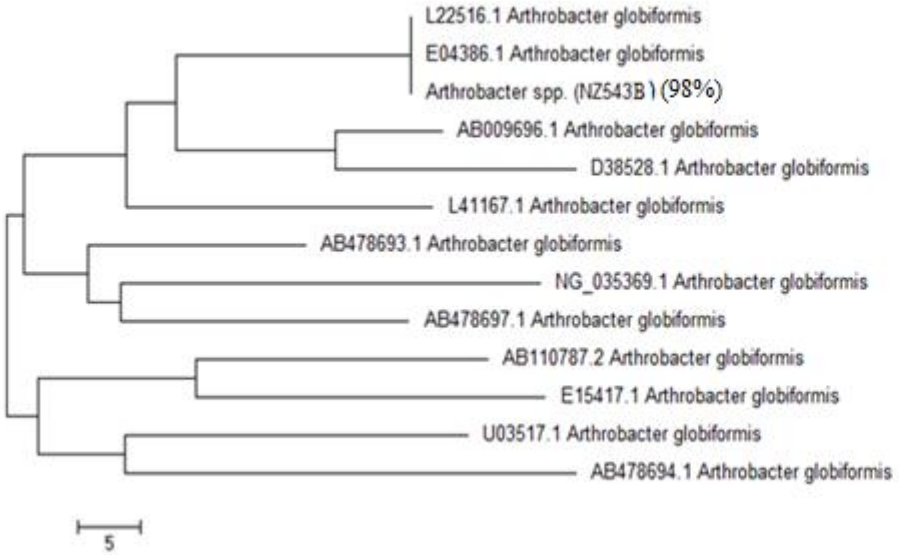


Figure 15: Phylogenetic tree of isolate NZ543B

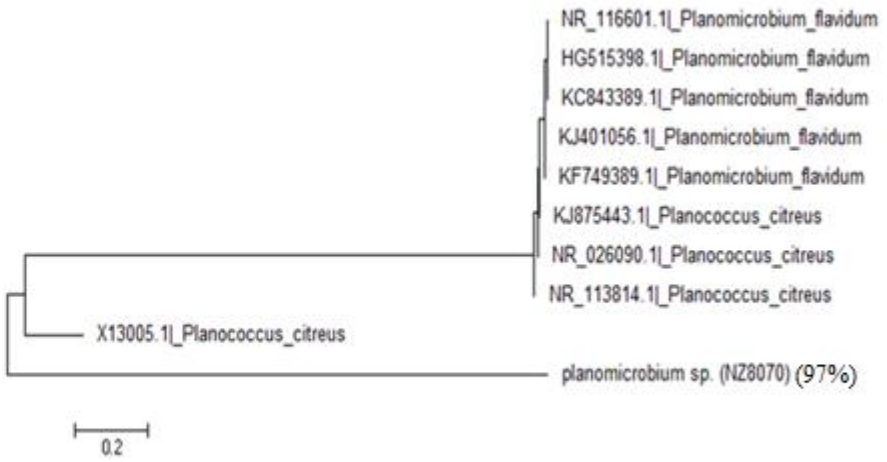


Figure 16: Phylogenetic tree of isolate NZ8070

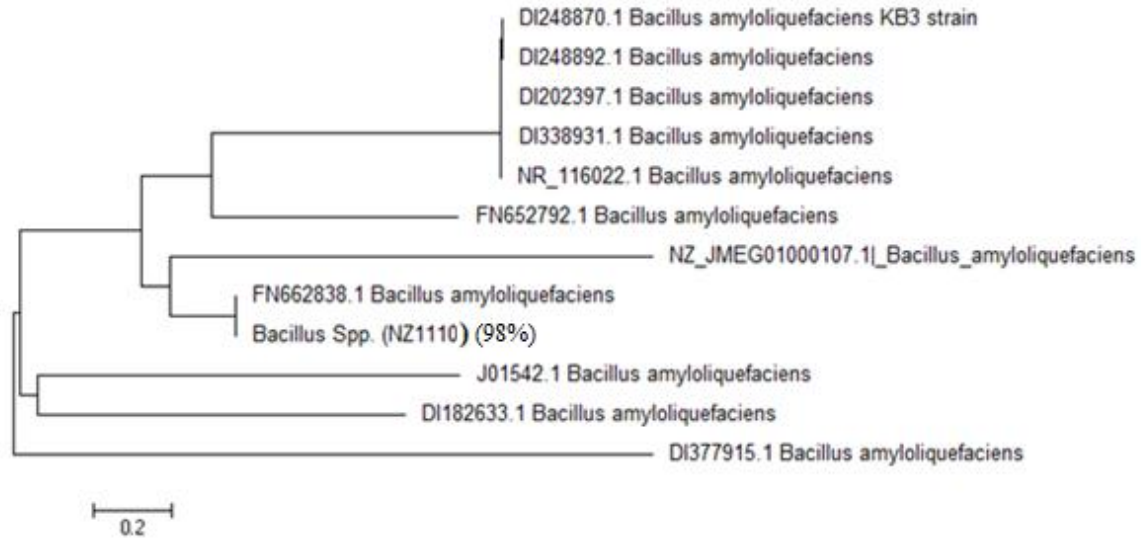


Figure 17: Phylogenetic tree of isolate NZ1110.

Table 15: Identified metribuzin degrading soil bacteria and their accession numbers.

Bacteria code	Species name	Accession numbers
NZ453C	*Pseudomonas sp.*	KX214668
NZ453B	*Burkholderia sp.*	KU937113
NZ453A	*Staphylococcus sp.*	KX394232
NZ543A	*Bacillus sp*	KX214666
NZ543B	*Arthrobacter sp*	KX394231
NZ1110	*Bacillus sp.*	KX394233
NZ8070	*Planomicrobium sp.*	KX214667

Related study by Gouma (2009) isolated nineteen *Bacillus sp* and four *Pseudomonas sp* which exhibited tolerance to metribuzin and a mixture of other pesticides. According to Abo-Amer (2012), *Pseudomonas sp*. is the most efficient bacteria genus for the degradation of toxic compounds. Zhang *et al.* (2014) reported isolation of *Bacillus sp.* which degraded 20mg L^{-1} of metribuzin, at a rate of 73% in 120hrs. In the current study similar genera of bacteria were isolated that also showed ability to degrade metribuzin. *Arthrobacter globiformis, Pseudomonas sp.* and *Bacillus sp.* are some of the bacteria described by Krutz (2010) as having triazine degrading genes. Metribuzin is a triazine and the current study has isolated some similar genera of bacteria as described by Krutz (2010). *Stapylococcus sciuri* and *Planomicrobium sp*. have not been reported as metribuzin degraders in previous studies. The two bacteria have been reported to be degraders of other compounds as described by

Vennila and Kannan (2010) and Mrozik and Labuzek (2002), respectively. Vennila and Kannan (2010) screened *Planomicrobium sp*. for hydrocarbon degradation and bioremediation and the bacteria showed capability to utilize kerosene as carbon source in minimal medium. Biological treatment of the refinery effluent with the *Planomicrobium sp.* reduced the oil and grease sulphide content to about 91.2% and 28% respectively on the 4th day of incubation.

CHAPTER FIVE
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Soils of Nzoia sugarcane farms contain varied concentrations of culturable bacteria and among them, a few degraded metribuzin. Total viable bacteria in Nzoia sugar company farms was more influenced by pH more than soil temperature and moisture separately. Metribuzin degraders isolated from Nzoia sugar farms are specifically different as observed in characterization.

Five of metribuzin degrading bacteria isolated from Nzoia sugarcane farm soils recorded highest growth at pH 9 and two of them with highest growth at pH 7. In multiplication of these bacteria in consideration for bioremediation, growth media can be adjusted to pH 9, to achieve higher number of bacteria cells. *In situ*, liming of soils to alkali pH can be considered to enhance favourable pH for these bacteria to thrive in the soils. Six of metribuzin degrading bacteria from Nzoia Sugarcane farms had highest growth at 35°C, while one had highest growth at 20^{o} C. For multiplication purposes 35 °C can be used for relatively higher multiplication of cells. The Isolates required addition of nitrogen and phosphorous in media for better growth, but generally higher growth was in higher concentrations of phosphorous than in nitrogen. For the purposes of multiplication of these bacteria additional nitrogen and phosphorous can be incorporated in media, but relatively higher concentration of phosphorous than nitrogen is required. *Insitu* measurements of phosphorous and nitrogen can be carried out to amend the soils in cases of deficiency if phosphorous and nitrogen levels should be considered during bioremediation.

Metribuzin degrading bacteria of Nzoia sugar farms were diverse in cell and cultural morphology, and biochemical reactions. *Planococcus sp., Burkholderia cepacia, Pseudomonas sp., Bacillus amyloliquefaciens, Bacillus pseudomycoides, Arthrobacter globiformis and Staphylococcus sciuri* are potential metribuzin degrading bacteria found in Nzoia sugar company farms. Metribuzin degrading bacteria of Nzoia sugarcane farms had high capability of degrading metribuzin producing various metabolites. Desamino-metribuzin (DA), Desamino-Diketo-metribuzin (DADK) and Diketo-metribuzin (DK) were the metabolites of metribuzin degradation, by bacteria isolates of Nzoia sugar company farms.

5.2 Recommendations

1. The metribuzin degrading bacteria obtained in this study could be used to formulate effective organisms for bioaugmentation trials.
2. In bioremediation process, liming of soils should be done because most of the degraders had highest growth in alkaline media. Liming increases soil pH and as Rousk *et al.,* (2009) reported in his study, increased pH enhances availability of nutrients hence increased bacterial growth.
3. In multiplication of the degraders, 35^{o}C should be used as incubation temperature and phosphorous should be added into the medium in higher concentrations than Nitrogen.
4. The study recommends further study using a consortium of microbes isolated since each may have different enzyme system and capacity to degrade metribuzin may be increased with use of different bacteria rather than one.
5. There would be need for further study to identify other possible metabolites of metribuzin degradation by the isolates of the study.

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