

**MICROBIAL DEGRADATION OF CHLORPYRIFOS RESIDUES AND THEIR
EFFECTS ON CALCIUM LEVELS IN FRESH AND PROCESSED MILK
IN NAKURU COUNTY, KENYA**

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DECLARATION

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

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DEDICATION

This work is dedicated to my wife, Sarah Moraa, my mother, Rhoda Asamba, and my siblings, Sam Asamba, Pacificah Asamba, Joshua Asamba, and Enoch Asamba, who have been supportive in my education life.

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ACRONYMS AND ABBREVIATIONS

Ach	Acetylcholine
AchE -ase	acetylcholinesterase
ANOVA	Analysis of variance
CFU	Colony Forming Unit
CNS	Central Nervous System
CP	Chlorpyrifos
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DMTP	Dimethyl thiophosphate
EC	Emulsifiable Concentrate
GC-MS	Gas Chromatography-Mass Spectrometry
GDP	Gross Domestic Product
GPC	Gel permeation chromatography
HCH	Hexachlorocyclohexane
HPLC	High Performance Liquid Chromatography
KIA	Kligler iron agar
LAB	Lactic Acid Bacteria
MR-VP	Methyl Red / Voges-Proskauer
MRL	Maximum Residue Limit
MSM	Minimum Salt Medium
MSPD	Matric solid-phase dispersion

NA	Nutrient Agar
OD	Optical Density
OP	Organophosphate
PCR	Polymerase Chain Reaction
MG/L	Parts per million
SCA	Simmons Citrate Agar
SPE	Solid Phase Extraction
SPME	Solid-phase micro extraction
TBC	Total bacteria count
TCP	Trichloro pyridine-2-phenol
UHT	Ultra Heat Treatment
UV-VIS	Ultraviolet–Visible Spectroscopy
WHO	World Health Organization

ABSTRACT

Acaricides are a class of pesticides used to control mites and ticks. The most widely applied acaricides belong to the organophosphorus (OP) group, including chlorpyrifos (CP) and Diazinon, which have high to moderate toxicity. They accumulate in animal tissues, milk, and other animal products, becoming a threat to human health. The presence of these residues and their metabolites in milk also alters milk composition and chemistry, including vital elements such as calcium. Some microorganisms can use acaricides as a source of energy and carbon, thus serving a critical role in bioremediation. The aim of the study was to determine chlorpyrifos levels in milk and milk products in Nakuru County and to isolate bacteria with CP degradation potential. A pre-study survey showed that dairy farmers in Nakuru County use various acaricides to control ticks, including the organophosphates chlorpyrifos and diazinon. Milk, water, soil, and dipwash samples were collected from selected farms in the county. High-Performance Liquid Chromatography was used to quantify CP residue levels in the collected samples. Approximately 53 % of raw milk samples had significant detectable chlorpyrifos levels, but no residues were detected in processed milk or high-value milk products. All the positive milk samples exceeded the maximum residue limit (MRL). The positivity rate in soil, dip wash, and water samples were 72.7 %, 62.5 %, and 27.3 %, respectively. No significant correlation was found between CP residues and total bacterial colony counts ($r = 0.019$; $p = 0.952$) in milk. Calcium levels in the milk samples were within recommended levels. A weak and negative correlation was found between calcium concentration and chlorpyrifos residue levels ($r = -0.2$, $p = 0.636$). Using enrichment culture technique, in Minimum Salt Medium (MSM) containing varying concentrations of chlorpyrifos (5 mg/l, 10 mg/l, and 40 mg/l), 18 isolates were found capable of degrading chlorpyrifos and coded MA1 to MA18. The degradation products were detected and identified by gas chromatography-mass spectrometry. There were no significant differences in growth among the three concentrations of chlorpyrifos used. The isolates were subjected to cultural, morphological, biochemical, and molecular characterization. Gene sequence analysis of 16S rRNA showed that the isolates with a potential to degrade and utilize chlorpyrifos as the sole carbon source belonged to the genera *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, *Alicialigenes*, *Lysinibacillus*, and *Achromobacter*. GC-MS analysis showed that the main degradation product was 2-Hydroxy-3,5,6-trichloropyridine (TCP). Significant CP degradation was reported in four isolates: MA1 (87.16 %), MA2 (82.04 %), MA4 (89.53 %) and MA8 (91.08 %). The findings show that soil in dairy farms in Nakuru County consists of different chlorpyrifos degrading bacteria, which grow in different physical-chemical conditions. The isolated strains can be multiplied and developed for bioremediation and biodegradation of organophosphate-contaminated soil and water.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Excessive exploitation of natural resources and the synthesis of xenobiotic compounds have led to the disruption of biogeochemical cycling, contamination of terrestrial and aquatic ecosystems, and harmful effects on various living organisms (Aslam *et al.*, 2013). Pesticides are toxic substances released intentionally to kill or retard the growth of pests of livestock and crops (Martins *et al.*, 2013; Jawaid *et al.*, 2016). So far, the most widely applied and popular pesticides are those that belong to the organophosphorus (OPs) group (Muñoz-Quezada *et al.*, 2013), which account for around 38 % of all the pesticides used worldwide (Ambreen and Yasmin, 2021). Their popularity is partly due to their short persistence and degradability (Sapahin *et al.*, 2014).

In modern agricultural production, more than 800 different types of pesticides are used to control fungi, rodents, insects, and unwanted plants (Aslam *et al.*, 2013). Most of these chemicals leave their residues in the environment, and they can be transferred up the food chain to humans (Kotinagu and Krishnaiah, 2015). Also, in animal husbandry, acaricides are used to prevent infestation by pests. As a result, there is bioaccumulation of persistent pesticides in animal products such as milk, meat, and eggs (Bajwa and Sandhu, 2011). In humans, pesticides have contributed to severe diseases such as cancer, heart disease, and Alzheimer's disease (Iftikhar *et al.*, 2014). Due to its lipophilic nature, milk is a key item for acaricide accumulation (Shaker and Elsharkwawy, 2015).

Synthetic organophosphate compounds are used in acaricides and insecticides to control and prevent parasitic diseases (Muñoz-Quezada *et al.*, 2013). Examples of organophosphates commonly used in dairy farming include chlorpyrifos (CP), diazinon, Ethion, and dichlorvos. Some are fat-soluble and therefore enter the body through the skin, eventually finding their way to the meat or milk products (Deti *et al.*, 2014). Pesticide residues in milk are linked to severe health conditions for humans (Jawaid *et al.*, 2016).

Lactating cows can be exposed to OP through many ways, including application on the animal body, ingestion of feed, fodder, and water, and in animal processing areas (Bedi *et al.*, 2015). Cow Milk is an excellent natural product consumed around the world as a rich source of proteins, fats, energy, and minerals and, thus, contains all the nutrients required for growth (Wanjiku *et al.*, 2022). Due to its universal intake, contamination of milk is a severe source of concern for public health (Shaker and Elsharkwawy, 2015).

Various studies have considered how to manage OP residues, including microbial degradation (Bose *et al.*, 2021). For instance, Kumar *et al.* (2013) demonstrated that intestinal lactic acid bacteria (LAB) efficiently degraded organophosphates. However, few studies have evaluated the biodegradation of OPs in Kenya, despite their widespread use. Removal toxic chemicals from the environment is desired because of their impacts on human health.

Research evidence shows that some microorganisms possess the ability to use pesticides as a source of energy and carbon (Willian, 2015). For instance, Singh and Walker (2006)

reported some fungi such as *Phanerochaete chrysosporium*, *Trametes hirsutus*, *Cyathus bulleri*, and *Phanerochaete sordida* could degrade lindane. It has been established that soil bacteria are the principal organisms for enhanced bio-degradation (Cycon *et al.*, 2017). Pure bacterial isolates that use specific organophosphates as a sole source of nitrogen, carbon, or phosphorus have also been identified (Briceno *et al.*, 2020). In some cases, OPs are degraded by mixture of bacteria, but individual organisms cannot utilize the acaricide as the energy source (Cao *et al.*, 2018). For some chemicals, microorganisms capable of using them for growth have not been isolated (Kumar *et al.*, 2018). This failure may not be used to conclude that there is no biological involvement in degradation since it can be due to other factors such as the composition of the media and environmental conditions, as some strains require certain growth factors; thus, they may be non-culturable (Hamsavathani *et al.*, 2017).

In Kenya, few studies have identified the indigenous organophosphate-decomposing microorganisms in soil and the laboratory. A survey of pesticide residues, their degradation, and their effect on calcium levels in milk will thus be of importance to the dairy and food industries. Therefore, the present study has been undertaken to assess CP residue levels in milk and their effects on calcium levels, and to isolate CP-degrading bacteria.

1.2 Statement of the Problem

Milk and milk products are an essential part of human nutrition worldwide, contributing a large portion of proteins in the diet (Bedi *et al.*, 2015). Therefore, milk must be of high hygienic quality without any hazardous chemical or microbial contamination. However,

the excessive use of pesticides and acaricides has led to retaining residues in milk and its processed products (Chen *et al.*, 2014). Considering the importance of organophosphates in agriculture, their use, persistence, and toxicity, there is a need for a comprehensive study on the effects of the metabolites formed in the pathways of the degradation process of the OPs. Previous studies have explored the impact of acaricide residues on various milk components, including proteins, carbohydrates, and lipids (Brancato *et al.*, 2017). Still, no study has examined how the residues and their metabolites affect essential minerals.

The estimated acceptable daily intake (ADI) of chlorpyrifos for humans, according to the WHO, is 0 to 0.01 mg/kg bw and the acute reference dose (ARfD) at 0.1 mg/kg bw (WHO, 2008; Mie *et al.*, 2018). For promoting trade and protecting consumers, maximum residue levels (MRLs) have been set for pesticides in products of animal origin, including milk (Shaker and Elsharkawy, 2015). In milk products, residue levels should not exceed 0.001 mg/l (Brancato *et al.*, 2017). However, contaminated dairy milk can contain amounts higher than this threshold, a severe health issue. Bundotich and Gichuhi (2015) reported that pesticides were some of the sources of acute poisoning reported in Nakuru County. Thus, OP residues in milk are a public health concern because dairy products are widely consumed in Kenya. Few studies in Kenya have examined the levels of Organophosphates such as Chlorpyrifos in milk and milk products. Thus, the present study will address this gap in the literature by determining OP residue levels in milk obtained from Nakuru County, Kenya.

Despite the widespread application of OP acaricides in Kenya, including Chlorpyrifos (Commercially sold as Duodip) and diazinon, few studies have been conducted to isolate and characterize microorganisms with degradation potential. Nakuru County is one of the significant milk-producing regions in Kenya, where both high intensity and low-intensity dairy farming are carried out (Lanyasunya *et al.*, 2006; Wangu *et al.*, 2021). However, few studies have so far explored the use of acaricides in Nakuru County, their retention in milk products, as well as the impact on calcium levels (Wanjiku *et al.*, 2022).

1.3 Justification

The application of pesticides in farming and animal husbandry is critical to overcoming the challenge of food insecurity (Deti *et al.*, 2014; Ambreen and Yasmin, 2021). Numerous studies have also reported on the health hazards associated with accumulation of pesticides in the food chain (Aslam *et al.*, 2013; Abdel-Wareth and Abd El-Hamid, 2016; Farhan *et al.*, 2021). In Kenya, the government promotes acaricides to control ticks in dairy animals, although they have known negative consequences to the environment (Omwenga *et al.*, 2020; Wanjiku *et al.*, 2022). The present study assesses the presence and levels of OP residues in milk, water, dip wash, and soil, which provides a clear picture of the impact of excessive use of acaricides. Besides, the study examines how the presence of CP residues affects the levels of Calcium in milk, which is a novel contribution to the impact of contaminants on milk composition.

A microbial remediation is a viable option for reducing the accumulation of these pesticides in the environment. In other words, despite the compelling evidence about the adverse health effects and lack of stringent implementation policies of government policy

in Kenya, the accumulation of the OPs generally, and chlorpyrifos specifically, will continue. Therefore, there is an urgent need to isolate and characterize microbial consortium capable of total degradation CP residues in the environment. It is envisaged that this would be a viable alternative and novel method of bioremediation of the contaminated environment. Consequently, it is necessary to investigate whether the use of CP acaricides leads to the persistence of residues in the background and whether the residues find their way to milk and milk products. The assessment forms a basis of assays to isolate and characterize microorganisms with the potential to degrade these residues.

The choice of Nakuru County is justified because the region is one of the leading dairy production areas in Kenya, where many farmers practice semi-zero grazing, in which cows graze during the day and they are provided supplementary feed and shelter at night (Wairimu *et al.*, 2021). Recently, there has been a steady rise in milk production in Nakuru County, due to adoption of intensive agricultural practices, including the use of acaricides to control ticks (Mureithi, 2019). It is, therefore, necessary to explore whether the use of acaricides leads to their entry into the food chain by appearing in milk products in the county.

1.4 Hypotheses

- i. There are no significant chlorpyrifos residues in milk, soil, farm water, and dip wash samples from Nakuru County.
- ii. There are no bacteria in soil, milk, water, and dip wash with chlorpyrifos-degrading potential.

- iii. There is no correlation between chlorpyrifos residues levels and calcium levels in milk samples.

1.5 Objectives

1.5.1 General objective

To assess the presence of chlorpyrifos residues in milk products and correlate with calcium levels and to isolate and characterize bacteria with CP-degrading potential from selected farms in Nakuru County, Kenya.

1.5.2 Specific objectives

- i. To determine chlorpyrifos residue levels in milk, soil, water, and dip wash samples from Nakuru County, Kenya.
- ii. To determine the diversity of chlorpyrifos-degrading bacteria in soil and dip-wash samples from Nakuru County, Kenya
- iii. To examine effect of chlorpyrifos residues on calcium levels in milk from Nakuru County, Kenya.

1.6 Significance of the Study

The study has assessed the presence and levels of chlorpyrifos residues in milk, milk products, soil, water, and dip wash in dairy farms from Nakuru, Kenya. The findings of this study are critical to the dairy industry, the food industry, and public health due to their ramifications concerning the fate of acaricides used to spray dairy animals, their accumulation in the environment, their presence in food products, and possible effects on human health. Therefore, the results of this study will inform future policy-making decisions on the proper use and disposal of chemical pesticides.

The study also sought to isolate and identify bacteria with the potential for degradation of chlorpyrifos. Assessing the degradation ability of bacterial strains from milk contaminated with OP pesticides will help in bioremediation since microbes utilize various compounds, including acaricides, for their growth and survival. The study is significant to the dairy and food industries since microbial bioremediation will offer an efficient milk decontamination mechanism (Yadav *et al.*, 2015). Understanding the means for degradation makes it possible to develop technologies to increase the efficiency of degradation, such as designing a bioreactor that degrades organophosphates using a bacterial consortium. According to Abdel-Wareth and Abd El-Hamid (2016), microbial degradation (bioremediation) is a critical and efficient method of removing pesticides from the environment. This study is significant as it empirically contributes to the potential of some bacterial species and consortia to degrade OP residues in the background. Biotic degradation is desired because of its low cost and minor collateral damage to indigenous organisms (Chishti *et al.*, 2013). The current study is among the first to explore biodegradation of CP using native bacteria isolated from soils and dip wash from dairy farms in Nakuru County, Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pesticides

A pesticide is a chemical used to control pests and vectors on crops, livestock, and human beings (Sahin *et al.*, 2017). Pesticides used for controlling animal pests, such as ticks and mites, are known as acaricides (Brito *et al.*, 2011). Most of them are toxic pollutants when released into the environment, which can harm people as they are being applied or consumed by contaminated food (Wanjiku *et al.*, 2022). Pesticides often get into the air, soil, water, and organisms living in the surrounding areas, eventually affected non-targeted organisms and plants. They may accumulate in levels that can cause harm (Sahin *et al.*, 2017). For instance, when dairy cows are fed on forages and water polluted by pesticides, the milk they produce may retain the pesticide residues (Bo *et al.*, 2011). Acaricides include organochloride compounds, organophosphorus compounds, carbamates, pyrethroids, ivermectins, and formamidines (Brito *et al.*, 2011).

According to Marete (2021), the use of pesticides in developing countries is not efficient because of many factors, including lack of qualified extension workers, application equipment, and knowledge, in addition to pest resistance and poor infrastructure. In Kenya, various classes of pesticides are used for agriculture, horticulture, and livestock production. Table 2.1 shows the commonly used acaricides in Kenya, their trade names, and chemical families (Mutavi *et al.*, 2021).

Table 2.1: Commonly used acaricides in Kenya

Trade Name	Active Ingredient	Chemical Family
Duodip 55EC	Chlorpyrifos	Organophosphate
Dursban 4EC	Chlorpyrifos	Organophosphate
Bulldock	Cypermethrin	Pyrethroid
Cyclone 505EC	Cypermethrin	Pyrethroid
Farm-X	Deltamethrin	Pyrethroid
Dimeton 40EC	Dimethoate	Organophosphate
Damadim 40EC	Dimethoate	Organophosphate
Lannate 90	Methomyl	Carbamates
Agrinate	Methomyl	Carbamates
Polytrin	Profenofos/Cypermethrin	Organophosphate/Pyrethroid
Selecron 720EC	Profenofos	Organophosphate
Steladone	Chlorfenvinphos	Organophosphate
Bye	Amitraz	Formamidines
Triatix	Amitraz	Formamidines
Alfapor	Apha-cypermethrin	Pyrethroids
Delete	Deltamethrin	Pyrethroid
Ectomin	Cybermethrin	Pyrethroid

2.2 Organophosphate Pesticides

Organophosphate pesticides (OPs) are synthetic biogenic compounds with a binding covalent bond between carbon and phosphorus that replaces a carbon-to-oxygen-to-phosphorus bond in a normal phosphate ester (Kazemi *et al.*, 2012). They are widely used in Agriculture around the world, including in Kenya. Various research studies have confirmed that exposure to OP pesticides can lead to adverse health effects (Kazemi *et al.*, 2012). For instance, it has been shown that those who apply these pesticides show signs of cognitive disorders, impaired nervous system function, development of lung and breast cancers leukemia, among other conditions (Kiljanek *et al.*, 2013; Marete, 2021).

Singh and Walker (2006) traced organophosphate history to 1937 when the first OP insecticide known as tetraethyl pyrophosphate was used. OPs have since been produced and used to control household and agricultural pests, with estimates showing that they

account for more than 38% of all pesticides sold worldwide (Timothy, 2011). The most commonly used OPs are diazinon and Chlorpyrifos. Others include parathion, diazinon, methyl parathion, coumaphos, malathion, and fenamiphos. OPs are preferred because they are less persistent in the environment and more effective in controlling pests than Dichlorodiphenyltrichloroethane (DDT) and lindane (WHO, 2008). According to Ambreen and Yasmin (2021), residues of OPs persist in water, air, and soil for a long time, resulting in biomagnification up the food chain.

2.2.1 Chlorpyrifos

Chlorpyrifos (0, 0 – diethyl 0 - (3, 5, 6 – trichloro – 2 - pyridyl phosphorothioate) is the most widely used OP pesticide in the world (Ambreen and Yasmin, 2021). The chemical's molecular formula is $C_9H_{11}Cl_3NO_3PS$, and the structural formula is shown in Figure 2.1 (European Chemicals Agency, 2020). It is effective against various insect pests of crops and livestock. It first came into the market in 1965, and ever since, it has been used in agriculture and for the control of mosquitos (US EPA, 2018). It is available in various brand names, such as Duodip, commonly used as an acaricide in Kenya. Its extensive use has led to water, air, rivers, and animal products (Solomon *et al.*, 2014). In some cases, the contamination is as far as 20 kilometers from the site of application. Chlorpyrifos (CP) has a half-life of between two and four months in the soil, although it can take over a year depending on the climate and soil type (Ambreen and Yasmin, 2021).

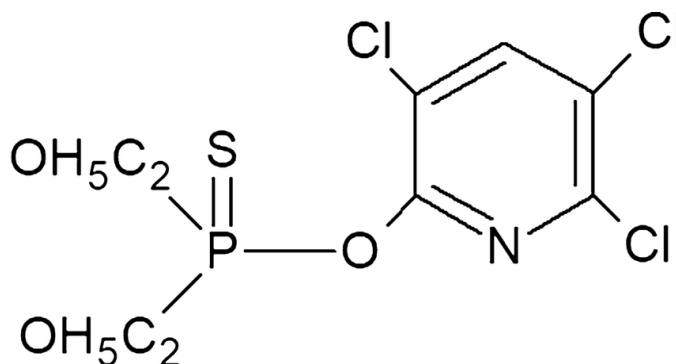


Figure 2.1: Chemical Structure of Chlorpyrifos (European Chemicals Agency, 2020)

The mechanism of action of Chlorpyrifos (CP) is related to its oxon metabolite, which combines with acetylcholinesterase (AChE-ase) in target tissues. Hence, it is an inhibitor of the enzyme, which is critical for the functioning of the nervous system in animals (Harishankar *et al.*, 2013). Typically, the AChE-ase enzyme is used to cancel the stimulating effect on the neurotransmitter chemical acetylcholine (Ach). When CP and other OPs inhibit the enzyme, it leads to sustained high levels of acetylcholine, which severely disrupts the Central Nervous System (CNS) activity. CP also targets other molecules such as RNA and DNA, interfering with their synthesis and expression. Cases of severe poisoning in humans by CP are known to cause symptoms such as nausea, headache, muscle twitching, dizziness, increased sweating, weakness, and salivation (Ambreen and Yasmin, 2021).

The widespread use of CP in agriculture is a cause for public health concern. According to Harishanker *et al.* (2013), CP can enter the human body through the mouth, skin, and lungs (Barathidasan *et al.*, 2014). When absorbed through the gut, it gets to the bloodstream and travels to the liver, where it is metabolized, resulting in liver toxicity. It can also be accumulated in body tissues, bones, fats, and proteins. Chlorpyrifos is

moderately toxic with acute oral LD₅₀; 135–163 mg kg⁻¹ for rat and 500 mg kg⁻¹ for guinea pig (Singh and Walker, 2006). In Kenya, CP has been listed as one of the pesticides recommended for withdrawal from the market. However, it is still the most used pesticides by dairy farmers in Nakuru County (Marete, 2021).

According to Ambreen and Yasmin (2021), the overuse of Chlorpyrifos in agriculture has led to deposition in water, soil, and food resources, resulting in biomagnification in non-target organisms, including humans. Toxic effects of this chemical have been reported, including on testes of male rats, where they cause a decrease in the number of sperms. In Kenya, the most common formulation of Chlorpyrifos is Duodip 55% EC, an ectoparasiticide used as a Spray or in Dips to control fleas, ticks, lice, and mange in camels, goats, sheep, pigs, cats, cattle, and dogs (Imagine Care Limited, 2020). The product contains Chlorpyrifos 500g/l and Cypermethrin 50g/l, being a combination of organophosphorus and synthetic pyrethroid. Before application, it is usually diluted with a lot of water. The manufacturers precaution that the product is toxic to humans, and it enters the body through the skin, eyes, or mouth. The WHO (2019) classifies Chlorpyrifos as a moderately hazardous chemical (Class II).

2.2.2 Diazinon

Diazinon (diethoxy-[(2-isopropyl-6-methyl-4-pyrimidinyl) oxy]-thioxophosphan (Figure 2.2) is a broad-spectrum organophosphate insecticide that usually is a yellow-brown colored liquid with a unique odor (Ahmadifard *et al.*, 2019). When heated, it generates poisonous gases (Wu *et al.*, 2021). It has low solubility in water; thus, it cannot penetrate far into the ground. It, therefore, remains in the soil for a long time, although

small amounts evaporate and decompose in the atmosphere in a few weeks (Pham and Bui, 2018). Furthermore, its emulsion is stable in hard water, a factor that contributes to its accumulation in aquatic organisms (Al-Otaibi *et al.*, 2019). The WHO classifies the chemical as a category II dangerous medium, with a lethal dose (LD50) for rats at 18mg/kg (Ahmadifard *et al.*, 2019).

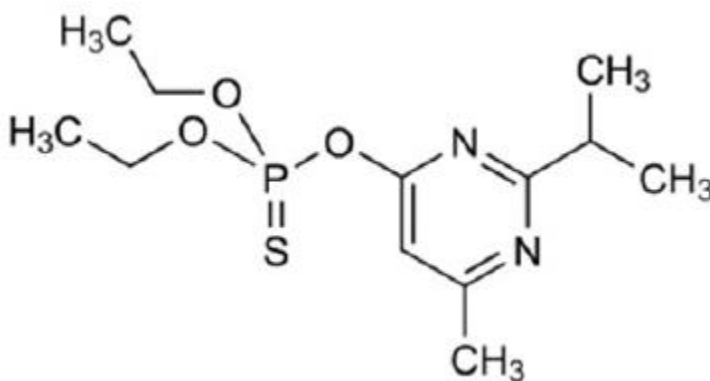


Figure 2.2: Chemical Structure of Diazinon (European Chemicals Agency, 2020)

2.3 Application of Acaricides in Dairy Farms

There is a growth of dairy farming production in Kenya, with the mixed farming system being used, including urban and peri-urban dairy farming (Rademaker *et al.*, 2016). In rural areas, mixed farming is the most dominant, especially by farmers with few livestock (Migose *et al.*, 2018). Most farmers in rural areas keep the animals in herds. Large-scale farmers stay large livestock numbers, either for dairy or meat production (Onono and Augustine, 2018). In Nakuru County, three types of dairy farming are carried out: High intensity, medium intensity, and low intensity. In all these cases, farmers use chemicals for controlling animal pests, including ticks (Jumba *et al.*, 2020).

The two main methods used to apply acaricides are spraying and plunge dipping (Brito *et al.*, 2011). Spraying is used for small and medium farms for various animals. Spraying allows for the use of highly concentrated acaricides, which are usually diluted in water. Three primary spraying techniques are used, including hand spraying, power spraying, and spray races. In Kenya, spray races are used in high and medium-intensity dairy farms to periodically control ticks and flies (Jumba *et al.*, 2020). For small-scale farmers, hand spraying with knapsack sprayers is preferred due to cost.

2.4 Presence of Organophosphate Residues in Milk and Milk Products

A cow's milk is a secretion obtained by standard milking methods from a lactating mammary gland of a cow. Milk is a complete food secreted to serve as a wholesome food for young suckling calves. Therefore, it has all the nutrients required for growth and development (Ishaq and Nawaz, 2018). It contains three essential components: water, fat, and non-fat solids (Avancini *et al.*, 2013). The primary organic matter comprises casein, albumin and globulin, lactose, and citric and lactic acids. Colostrum (the thick secreted milk after parturition) is slightly different in composition from regular milk. Also, a cow's milk responds differently to common indicators: acid to phenolphthalein; alkaline to methyl orange; and amphoteric to litmus paper. The normal pH of milk is between 6.4-6.6. Acidity increases on storage due to the action of microorganisms, especially the Lactic Acid Bacteria (LAB).

Milk is a vital source of macro-and micronutrients for improved nutrition of the population. In Kenya, dairy farming is one of the pathways out of poverty for many rural communities (Onono and Augustine, 2018). Due to population increase, urbanization,

and economic growth (Msalya, 2017). It is predicted that dairy farming contributes immensely to gross domestic product (GDP). In Kenya, the production of milk has increased by around 120% in the last ten years (Wairimu *et al.*, 2021). Dairy products are loosely nicknamed “white gold” due to their economic significance (Onono and Augustine, 2018). Although there is a significant contribution from exotic and hybrid animals, much milk is still obtained from local indigenous breeds. A small portion is obtained from camels, sheep, and goats. Most milk and milk products are sold informally through door-to-door sales, farm gates, open markets, and small shops by vendors (Onono and Augustine, 2018).

Although pesticides play an essential role in agriculture, they bring hazards to food safety and human health, as well as environmental pollution (Dordevic *et al.*, 2016). One of the main parts of the human diet nowadays is milk and its processed products such as cheese, yogurt, Malai, and butter (Dawood *et al.*, 2004). An increase in Milk production can be attributed to the intensive application of pesticides and acaricides to control livestock pests (Migose *et al.*, 2018). Broad-spectrum acaricides with extended insecticidal activity, such as organophosphates malathion and Chlorpyrifos , are the most commonly used (Sara *et al.*, 2019).

Due to lack of proper regulatory guidelines and poor management of residues after acaricide application, there is accumulation in the environment, especially through soil and water sources, eventually finding their way into milk and related products. Lactating cows accumulate pesticides by inhaling contaminated air and in contaminated feed and drinking water. Due to their lipophilic properties, organophosphates are often stored in

fat-rich tissues, from where they move to milk for excretion (Gill *et al.*, 2020). Therefore, milk consumption from exposed animals can lead to dire health consequences for humans. In some cases, the primary source of contamination is the presence of pesticide residues in animal feed (Wanjiku *et al.*, 2022). However, there are other sources of contamination, including acaricide application, environmental contamination, and accidental spills (Calahorrano-Moreno *et al.*, 2022). Recently, some studies have reported the presence of CP residues in milk, due to the ability of OPs to link covalently to the milk proteins (Gill *et al.*, 2020). Thus, residues in the feed can be consumed by the cow, and find their way to the milk in the udder.

2.5 Milk Processing Techniques that reduce Acaricide Residue Levels in Milk and Milk Products

According to Bajwa and Sandhu (2014), pesticides can be degraded by hydrolysis, photolysis, oxidation and reduction, pH, temperature, and metabolism. Organophosphate contaminated milk typically retains the residues in the fat portion, which leads to higher concentrations in some milk products such as butter, ghee, cheese, and malai (Calahorrano-Moreno *et al.*, 2022). Bajwa and Sandhu (2014) discussed the effect of preparation and processing on pesticide residues in milk. First, they reported that thermal treatment such as pasteurization and ultra-heat treatment (UHT) reduces the insecticide residues due to evaporation, co-distillation, and thermal degradation.

Moreover, Calahorrano-Moreno *et al.* (2022) investigated the decontamination of insecticide residues in milk by kitchen processes such as boiling, curd, forming ghee and butter, and removing malai. They found out that boiling leads to a decrease of

hexachlorocyclohexane (HCH) isomers by up to 26% and DDT by 35%. Soukoulis *et al.* (2007) found out that the manufacture of yogurt led to a decrease in pesticide residues by approximately 10%. Recent studies have found that sterilized milk contains lower residues than pasteurized milk, implying that the intensity of thermal treatment influenced pesticide degradation (Alegbeleye *et al.*, 2018).

2.6 Microbial Degradation of Organophosphate Compounds

Microbial communities are vital in controlling toxic chemicals in the environment because they can degrade these chemicals (Briceño *et al.*, 2020). Studies have reported greater bacterial diversity than fungi in the bio-purification system (Briceño *et al.*, 2020). It has been established that the degradation of pesticides in the soil is caused by microorganisms, which utilize the compounds as sources of energy and critical elements (Cycoń *et al.*, 2017) because they possess genetic adaptations that help them to synthesize enzymes that hydrolyze, oxidize, and hydroxylate the compounds. However, although single species play a role in transforming the toxic chemicals, complete mineralization of the residues is likely to be a result of mixed populations (Briceno *et al.*, 2020). Among the most commonly isolated bacterial genera responsible for the degradation of pesticides are the *Achromobactor*, *Arthrobactor*, *Streptomyces*, and *Pseudomonas* (Briceño *et al.*, 2012; Chishti *et al.*, 2013).

OPs are esters with phosphorus present as either a phosphonate or phosphate ester. As such, these molecules possess sites with vulnerability for hydrolysis. Singh and Walker (2006) explained that microorganisms could degrade organophosphates by hydrolyzing

the P-O-alkyl and the P-O-aryl bonds leading to their detoxification. Various studies have identified different groups of bacteria that detoxify organophosphates through co-metabolism and bio-mineralization (Kumar *et al.*, 2018; Briceno *et al.*, 2020). Most studies, however, have focused only on detoxification without analyzing the metabolism of phosphorus-containing products (Cao *et al.*, 2018).

Many bacteria strains have been isolated and characterized with the potential to degrade OP compounds through hydrolysis, especially members of *Pseudomonas*, *Bacillus*, and *Aliccaligenes* genera (Kumar *et al.*, 2018). It has been shown that an increase in biodegradation activity is related to the expression of specific genes (Firozjaei *et al.*, 2015). Also, microbial growth and lateral gene transfer lead to an increase in the number of degrading microorganisms (Cao *et al.*, 2018). The third factor is the migration of degrading microorganisms to desired locations. Additionally, some organisms have evolved new essential enzymes for the breakdown of OPs.

Some bioremediation research studies have investigated the use of transitory food-grade bacteria to reduce the absorption of pesticide toxins. Particularly, lactic acid bacteria have been shown to reduce the contamination of OP in milk (Vasquez *et al.*, 2012). Zhang *et al.* (2016) investigated the biodegradation of two OP pesticides (chlorpyrifos and phorate) in whole corn silage by the bacteria *Lactobacillus. plantarum*. They analyzed OP residues in the silage weekly by using gas chromatography after extracting and purifying the OPs. They then calculated the degradation rate constants at different fermentation stages. The results showed that the microorganisms accelerated the degradation of the OPs. Further, they noted that a combination of strains led to increased degradation rates compared to using only one strain. Generally, there are two forms of

microbial degradation, catabolism, and cometabolism (Zhang *et al.*, 2016). Catabolism is a type in which there is the complete degradation of the organic chemical to produce energy and chemicals required for the growth of the microorganism. On the other hand, cometabolism is characterized by partial degradation, which does not typically benefit the organism (Góngora-Echeverría *et al.*, 2018).

Das and Adhya (2014) asserted that biodegradation of pesticides in the soil is influenced by both abiotic and biotic factors, which generally work together in the soil environment. Several physicochemical factors that include temperature, pH, organic carbon content, moisture content, and formulation of the acaricide influence the degradation. In this regard, Chlorpyrifos in the soil can be degraded by abiotic factors such as dechlorination, chemical hydrolysis, and UV light. Through hydrolysis, the chemical produces intermediate by-products, including Chlorpyrifos is 3,5,6-trichloro pyridine-2-phenol (TCP) and dimethyl thiophosphate (DMTP) (Naphade *et al.*, 2012).

2.6.1 Microbial Degradation of Chlorpyrifos

The European Chemicals Agency (2020) noted that anaerobic and aerobic biodegradation are notable Chlorpyrifos dissipation routes. In the soil, available data shows that the compound degrades slowly both under anaerobic and aerobic conditions. CP has been shown to have a high soil binding ability and low water solubility (European Chemicals Agency, 2020). According to Zhang *et al.* (2016), there have been few reports of enhanced CP degradation. Various studies have investigated microbial degradation of CP, although few have given an indepth analysis of enzymatic and genetic aspects of the degradation (Rayu *et al.*, 2017). Various studies have determined that microorganisms are

vital for the degradation of CP residues in the soil. Under controlled conditions, microbial enzymes can hydrolyze CP and mineralize the two main metabolites, 3, 5, 6- trichloro-2-pyridinol (TCP) and 3, 5 6-trichloro-2-methoxy pyridine (TMP) metabolites.

There is extensive literature about the environmental fate of CP. Singh and Walker (2006) reported that the half-life of CP in the soil is between 10 to 120 days. The significant variation is due to different environmental factors such as moisture content, temperature, and pH. The chemical does not undergo enhanced degradation as other OP compounds (Kashi *et al.*, 2021). This is due to the accumulation of TCP, a metabolite with anti-microbial properties which serves as a buffer to prevent the growth of CP degradation bacteria (Ambreen and Yasmin, 2021).

Previously, Ambreen and Yasmin (2021) reported that the degradation of Chlorpyrifos in alkaline soil was thought to be caused by hydrolysis. Still, later it was found that soil microorganisms are involved because sterile alkaline conditions do not lead to degradation. Since the presence of chloride radicals in the structure of Chlorpyrifos causes a reduction of water solubility, biodegradation is promoted by microbes that produce lipid biosurfactants and rhamnolipids for dissolving the chemical before degradation. Some bacteria possess the halogenase enzyme that removes chloride ions from Chlorpyrifos . In previous studies, some bacterial strains have been isolated with the potential to degrade Chlorpyrifos (Das and Adhya, 2015; Ferrario *et al.*, 2017). Bacterial species such as *Alcaligenes faecalis* and *Pseudomonas* sp. can mineralize TCP in the soil as well as in liquid medium (Ambreen and Yasmin, 2021).

2.6.2 Microbial Degradation of Diazinon

Wu *et al.* (2021) isolated and characterized diazinon degrading bacteria from contaminated soils. He used Bushnell-Hass medium to enrich the Diazinon-degrading bacteria. Using PCR and amplification of 16s rDNA, he identified and sequenced the prevalent degrading strains. The results show that there are some diazinon degrading bacteria in contaminated soils. They belong to *Pseudomonas putida* strain D3, *P. fluorescens* strain D1 and *Achromobacter piechaudii* strain D8. The decomposition was confirmed using gas chromatography (GC). Abo-Amer (2011) investigated to determine the biodegradation of diazinon by *Erratia marcescens* DI101. The strain was found to degrade other organophosphates such as parathion, coumaphos, and Chlorpyrifos.

2.7 Organophosphate Metabolites and Calcium Levels in milk

In recent years, dietary calcium has generated much research interest because of its vital role in preventing certain human conditions (Pettifor, 2014; Cormick and Belizán, 2019). Milk and milk products are rich in essential minerals and are responsible for about 70% of the calcium in the human diet. In this regard, milk products are significant in addressing calcium deficiency. It should be noted that around 2% of the human body weight is calcium, located in teeth and skeleton (Tunick, 1987). The deficiency of calcium in the human body is implicated in osteoporosis, hypertension, and amyotrophic lateral sclerosis (Cormick and Belizán, 2019). Eshpari *et al* (2016) suggested that the degradation of organophosphate pesticides can alter the composition of milk since the mineral equilibrium will be changed. The inorganic phosphates released combine with calcium ions to form inorganic salts. On average, a cow's milk contains 30mM of total calcium (Hidiroglou and Proulx, 1982).

2.8 Organophosphate Compounds and Human Health

Firozjahi *et al.* (2015) reviewed the health risks OPs pose to humans and found out that they affect multiple biological systems. The the most affected is the nervous system, through inhibition of cholinesterase activity leading to accumulation of acetylcholine at neurojunctions. The accumulation leads to changes in calcium ion concentration both inside and outside cells, thus affecting protein function in the endoplasmic reticulum. Secondly, Firozjahi *et al.* (2015) reported that some pesticides affect spermatogenesis and gametogenesis by disrupting endocrine function in humans and animals. Thirdly, OPs affect biological micromolecules involved in essential cell processes, such as the mitochondria, chromosomes, and proteins (Hossain *et al.*, 2013).

Further, it has been shown that the OPs lead to poisoning and related symptoms such as diarrhea, urination, tears, sweating, and excessive saliva production (Andersson *et al.*, 2014). OPs poison insects, birds, amphibians, and mammals through the phosphorylation of AChE at the nerve endings. This leads to overaccumulation of ACh, causing cholinergic poisoning (Hamid *et al.*, 2021). Muñoz-Quezada *et al.* (2013) noted that OP pesticides act as acetylcholinesterase (AChE) inhibitors, thereby preventing the breakdown of acetylcholine. Exposure of humans to these chemicals can be toxic, even at minimal levels (Hazarika *et al.*, 2020). They primarily affect infants more than adults because acetylcholine plays a significant role in the development of the brain. According to Ahmadifard *et al.* (2019), Diazinon causes reproductive growth failure, liver damage, weight loss in sexual organs, non-Hodgkin lymphoma, diabetes, and sperm abnormalities.

2.9 Methods of Organophosphates Detection and Quantification

Various methods have been used to determine pesticide residues in milk samples through separation and detection techniques. Solid-phase extraction (SPE) and matrix solid-phase dispersion (MSPD) are some of the most widely used techniques for cleaning the milk extraction for analysis (Kiljanek *et al.*, 2013). In recent years, solid-phase micro extraction (SPME) is an effective analytical technique (Garlito *et al.*, 2019). Moreover, Gel permeation chromatography (GPC) has traditionally been used for OP identification in animal products. According to Hongsibsong *et al.* (2020), most standard methods and techniques for detecting Chlorpyrifos are chromatographic based (liquid chromatography and gas chromatography). The methods often have complicated steps, use expensive equipment, and require technical help (Zhang *et al.*, 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site Description

The study was carried out in Nakuru County, located in the Rift Valley region of Kenya (Latitude: 0° 29' 59.99" N; Longitude: 36° 00' 0.00" E) (Figure 3.1). Nakuru is 1871 meters above sea level, with a mild climate, generally temperate and warm. Annual rainfall is approximately 762 mm, and the average temperature is 17.5 °C. Laboratory experiments were carried out at the Mt. Kenya University Research Center.

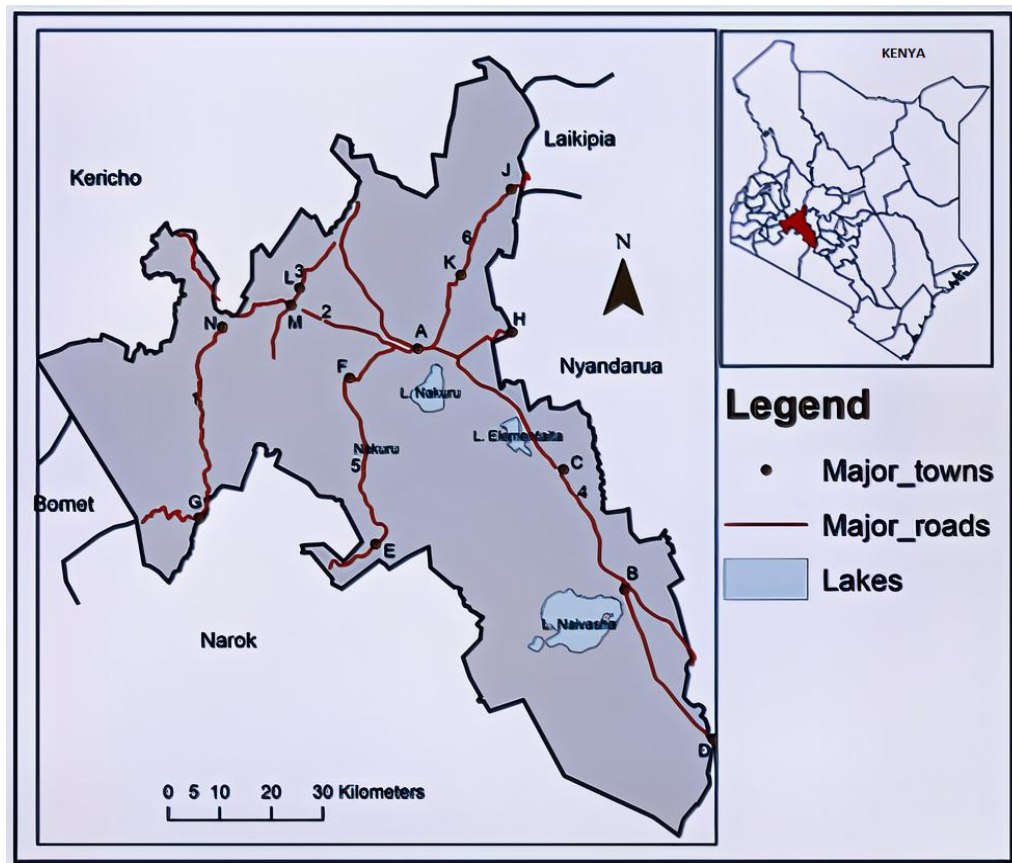


Figure 3.1: A map of the study site in Nakuru County, Kenya

3.2 Pre-study Survey

The use of acaricides baseline survey was done through a cross-sectional study design. Nakuru County was purposely selected because it was considered to be one of the leading producers of dairy products, with intensive livestock farming. A random sampling technique was used to select a sample of large-scale dairy farmers in the county. Collection of data was carried out using questionnaires (Appendix 1), personal observations, and face-to-face interview. The survey identified methods and frequency of acaricide application in Nakuru County.

3.3 Sampling Design

The study was carried out using a cross-sectional design. . The collection of samples was done in November 2018 and in November 2020. Fifteen dairy farms in Nakuru County were selected because they had a recent or current OP acaricide use history. Samples of milk, milk products (yogurt, cheese, *malai*), potentially contaminated soil, cattle dip waste (dip wash), and farm livestock drinking water were then collected. Samples were collected from five sub-countries in Nakuru County: Molo, Njoro, Rongai, Subukia, and Naivasha, based on stratified sampling, in which three farms were selected from each sub-county.

3.3.1 Milk Sample Collection

Fresh milk samples, 100 ml from each dairy farm, were collected directly from the farms immediately after milking, and they were kept in sterilized screw cap plastic bottles. Processed milk was collected from dairy processing companies within the County and retailers.

3.3.2 Soil Sample Collection

Soil samples were collected from dairy farms near cattle units, close to areas where animals are treated with acaricides (dips and spray races). The collection was done from topsoil, and stored in sterile sealable plastic zipper bags, in the refrigerator at 4 °C. The soil was picked at 10 cm below the soil surface. Physicochemical analysis was conducted for the soil because the source of the inoculum is related to bacterial properties. Properties of the soil measured include pH, electrical conductivity, soil water content, soil texture, and soil organic matter. In the laboratory, soil samples were air-dried and sieved before analysis.

3.3.3 Water and Dip Wash Collection

Water samples, 100ml from each site, were collected from the livestock drinking points within the dairy farms. They were kept in sterilized screw cap plastic bottles and placed in the cold box for transportation. Depending on the nature of acaricide application used in each farm, dip wash or spent spray were collected from the application points (Spray races and dips) and placed in sterilized screw cap plastic bottles.

3.4 Sample Handling, Transportation, and Storage

The collected samples were coded and stored in a cool box with ice packs during fieldwork. After that, they were transported to the Research laboratory at Mount Kenya University, where they were stored in a refrigerator at 4°C, until analysis. The second sampling was conducted in November 2020 in the same farms and regions. The sampling and collection procedures used were the same as those used in the first sampling. The second sampling was used to collate and augment the findings of the first sampling.

3.5 Chemicals and Reagents

The standard used in this analysis was Chlorpyrifos obtained from Sigma-Aldrich Corporation, having a purity of 99.5%. Commercial acaricides were purchased from an agrovet in Thika. The media used for Microbiological analysis were also obtained from Sigma-Aldrich, including MacConkey Agar, Nutrient Agar, Tryptic Soy Broth Medium, Simmons Citrate Agar, Kligler's Iron Agar, and Urea Agar Base. Bacteriological Agar and peptone water were also obtained from Sigma-Aldrich. All reagents used in the study were prepared by using the instructions of the manufacturers. All chromatography solvents including water and acetonitrile used in the analyses were HPLC grade (purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA). Adjustment of pH was done by using the Benchtop pH meter.

3.6 Chromatographic Identification of Organophosphate Residues

The protocol described by Mauldin *et al.* (2006) was used for Chromatographic analysis, with modifications. The identification and quantification of OP acaricides in the samples from Nakuru County was conducted by High-Performance Liquid Chromatography (HPLC) using Shimadzu HPLC with two LC-10AT VP pumps variable wavelength UV detector and a reversed-phase C-18 column (Shaker and Elsharkawy, 2015). The HPLC machine was equipped with the Lab Solutions software (Shimadzu). The mobile phase components (75 % acetonitrile and 25 % PO₄ buffer) were pumped to the column at a flow rate of one ml per minute at a temperature of 30°C. The UV was maintained at 230 nm, 20µl of samples were sequentially injected and analyzed by reverse-phase HPLC.

Retention times and peak areas were used to identify and quantify compounds in comparison with the reference standards.

The standard used in the analysis was Chlorpyrifos, which was dissolved in acetonitrile and serially diluted to make solutions of 10mg/l (Chen *et al.*, 2014). The linearity of the calibration curve was evaluated from peak area calculations after five injections of the standard solutions to make 2 mg/l, 4 mg/l, 6 mg/l, 8 mg/l, and 10 mg/l (Rayu *et al.*, 2017). Analyses were carried out in batches, using a pre-set method file saved in the machine software as the Dichloroquant method. Peak area was calculated for each sample, and the calibration curve (Figure 3.2) was plotted for concentration versus response area/peak area. The precision of the analyte was measured from the standard deviation, and an external standardization method with a standard deviation of less than 0.05 was used.

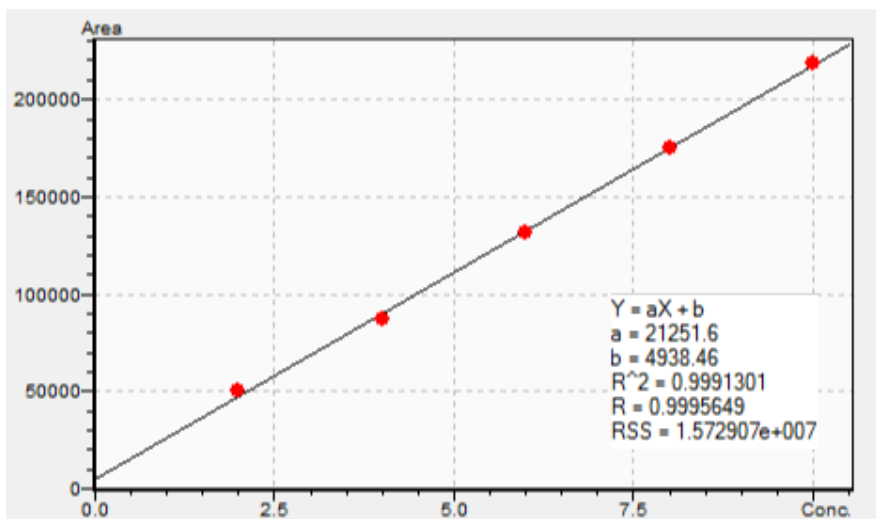


Figure 3.2: HPLC Calibration Curve of Chlorpyrifos Standard

The Chromatogram of the CP standard is presented in Figure 3.3. The retention time of the standard was 7.196 minutes. As such, samples that a peak with a retention time of ± 0.05 minutes of the standard were determined to be positive.

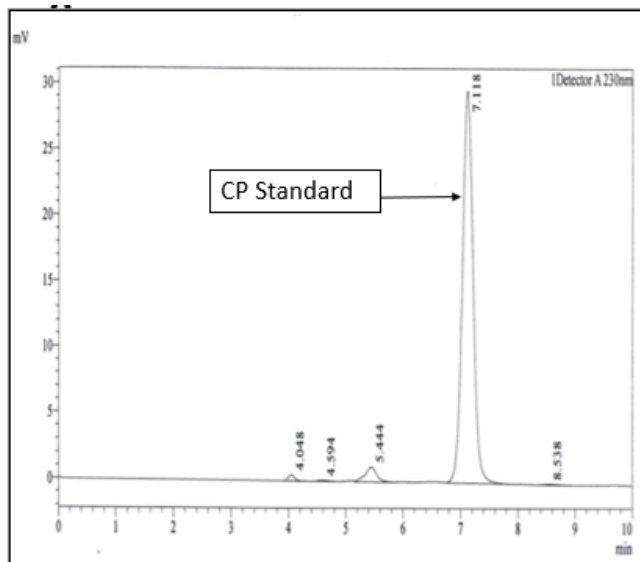


Figure 3.3: Chromatogram of the Chlorpyrifos Standard: Retention time at 7.1 minutes

3.6.1 Extraction Procedure

An optimized extraction method described by Fagnani *et al.* (2011) was used for all samples of milk, water, soil, and dip wash sampled from Nakuru County, with modifications. Samples were first defrosted, and 1ml of each sample was pipetted into a screw-top centrifuge tube. 5 ml of a solution of acetonitrile and phosphate buffer (90/10 v/v and pH 4.5) was added. The mixture was gently swirled, sonicated for 10 minutes, and vortexed for 15 minutes at the speed of 1,200 rpm. After that, centrifugation was done at 2000 rpm for 5 minutes. The supernatant was transferred into a screw cap test tube. Double extraction was done by adding 5ml of the premixed solution of acetonitrile and phosphate buffer to the original sample, and the procedure was repeated. The supernatant was added to the test tube. An aliquot of the extract was filtered through a

0.45µl Teflon syringe filter into an HPLC vial (Harshit *et al.*, 2017). The extraction and analysis were done in triplicates for each sample.

3.7 Estimation of Bacterial Population in Milk

For raw milk samples, bacteria estimation was done using the spread plate method as suggested by Rangaswami and Bagyaraj (2001). In this regard, 1ml of the sample was added to 9ml of saline water and diluted serially to yield six dilutions plated onto NA media, followed by incubation at 30°C for 24 hours. The number of colonies formed on the plates was enumerated to determine the number of colony-forming units (CFU) per milliliter of milk (McGrath *et al.*, 2015; Abera *et al.*, 2016). The CFUs in the present study were compared with the standards set by the European Union of total bacteria counts (TBC $\leq 1 \times 10^5$ CFU/ml) as acceptable minimum levels of microbial contamination in milk (Abera *et al.*, 2016).

3.8 Isolation of Chlorpyrifos Degrading Bacteria by Enrichment Culture

Technique

Enrichment culture technique using minimum salt medium was used to isolate potential Chlorpyrifos bacteria (Barathidasan *et al.*, 2014). The constituents of the Mineral Salt medium were (NH₄)₂NO₃, 1.0g; Ca (NO₃).2H₂O, 0.04 g; MgSO₄.7H₂O, 0.1 g; KCl, 0.2 g; FeSO₄.7H₂O, 0.001 g, K₂HPO₄.12H₂O, 1.5 g; and KH₂PO₄, 4.8 g. Adjustment of pH was made by using the Bench top pH meter. Commercial grade Chlorpyrifos was supplemented into the medium to serve as the only source of carbon. Chlorpyrifos supplementation was done at three different concentrations: 5 mg/l, 10 mg/l, and 40 mg/l. From each composite, a gram of soil was dispersed in 10 ml of distilled water and serially

diluted. Afterward, the sixth dilution (10^{-6}) was inoculated in MSM broth. The liquid media were incubated on a rotary shaker (150 rpm) (Figure 3.4) and maintained at 30°C and optimum pH for 21 days. Bacterial growth was assessed by measuring changes in medium turbidity (OD_{600}) on a UV-VIS Spectrophotometer. Samples of the liquid media were collected periodically (5 days) for Optical density measurement.

After 21 days, 1 ml aliquots were spread plated into MSM agar containing 10 mg/l Chlorpyrifos as the carbon source (Barathidasan *et al.*, 2014). After that, single colonies were obtained and resuspended in basal MSM with CP for 14 days to confirm the ability of the isolates to utilize the organophosphate. The cultures were then streaked on NA plates to obtain pure colonies. Colonies that were visually different were plated on Nutrient Agar (NA), and Tryptic Soy (TS) plates supplemented with Chlorpyrifos and incubated at 30°C. Cycloheximide was added to the media to suppress the growth of fungi. The isolates which had initially been grown in 10 mg/l Chlorpyrifos were in the acclimation period grown in 5 mg/l, 10 mg/l, 40 mg/l, and 100 mg/l. In the second set of experiments, fresh soil and dip wash/spray water samples were obtained from the field, and the same procedure above was utilized. The new samples were made into composites by grouping those from the same region to form three composites coded as N1, N2, and N3, with N referring to Nakuru. Letters S and D were used for soil and Dip-wash, respectively, leading to samples with codes as N1D, N1S, N2S, etc. Different concentrations of the organophosphate were used (5 mg/l, 10 mg/l, and 40 mg/l). Each sample was cultured in triplicates to minimize the chances of error.

3.8.1 Morphological and Microscopic Examination Bacterial Isolates

Preliminary characterization of the isolated bacteria was done using cultural and morphological characteristics. Colony characteristics used for bacteria identification include colour, elevation, margin, and size. A compound microscope was used to identify the isolates, aided by the gram staining technique (Becerra *et al.*, 2016).

On a clean, grease-free slide, a smear of suspension was made with a loopful of sample. It was air dried and heat fixed. Crystal Violet was flooded on the slide for one minute and rinsed with water. It was followed by pouring of the gram's iodine for a minute and rinsing with water. Afterward, 95% of acetone was used for 10 seconds, followed by rinsing with water. Finally, Safranin was added for a minute and washed with water. The slide was then air dried, blot dried, and observed under a compound microscope (Vincent *et al.*, 2021).

3.8.2 Biochemical Characterization of Potential Chloropyrifos Degrading Bacteria

Biochemical tests were conducted for preliminary identification of the isolates. Various cultural mediums were used. The results of these tests were vital for the presumptive identification of isolates before the phylogenetic analysis. The tests that were carried out included Methyl Red, Indole, Catalase, Oxidase, Citrate utilization, urease, and Kligler Iron Agar (KIA) test.

3.8.3 Molecular Characterization of Bacterial Isolates

The isolated bacteria were further characterized by using molecular techniques based on the 16S rRNA gene region (16S rDNA) sequencing. Four steps were followed in this

process, which were genomic DNA extraction, polymerase chain reaction (PCR) amplification, agarose Gel electrophoresis, and gene sequencing (Clarridge, 2004; Daneshparvar *et al.*, 2017; Galal and Seufi, 2020).

3.8.3.1 DNA Extraction

The Zymo quick DNA™ min prep kit (Zymo Research, USA) was used for genomic DNA extraction, with strict adherence to manufacturer instructions (Wei *et al.*, 2020). In this regard, freshly cultured bacteria isolates were suspended in normal saline (500 µL) in Eppendorf tubes. Afterward, vortexing was done for 10 seconds, followed by three minutes of centrifugation at 13,000 rpm. Subsequently, the sodium chloride was discarded, and genomic lysis buffer (400 µL) was added to the mixture, followed by further 10 seconds of vortexing. Incubation of the contents was carried out for 30 minutes at room temperature, which was followed by a transfer to the zymospin column placed in collection tubes and subjected to centrifugation for a minute at 13,000 rpm. Afterward, a pre-wash buffer was added (200 µL) followed by another round of centrifugation (Daneshparvar *et al.*, 2017). The next step was the addition of gDNA wash buffer (500 µL) and centrifuged at 13,000 rpm. Afterward, contents were transferred to micro-centrifuge tubes, and DNA elution buffer (50 µL) was added. After incubating the contents at room temperature for 10 minutes, final centrifugation was done for 30 seconds at 13,000 rpm, and the eluted DNA was kept at -20 °C (Cao *et al.*, 2018).

3.8.3.2 PCR Amplification

The protocol described by Lorenz (2012) was applied in the Polymerase Chain Reaction (PCR) for amplification of the bacteria 16S rRNA in a 20 well thermocycler. After the

initial denaturation at 95°C for 5 minutes, automated sequential 30 cycles were done that included denaturing at 95 °C for 45 sec, primer annealing at 62 °C for 45 sec, and extension at 72 °C for 2 minutes. The final extension was set at 72°C for 5 minutes. The obtained DNA was stored at 4°C. The primers used in the amplification of the 16S rRNA gene were the 1492r (5'GTTACCTTGTTACGACTTC-3') and 27f (5'-AGTTTGATCCTGGCTCAG-3').

3.8.3.3 Agarose Gel Electrophoresis

The PCR products were stained using SYBR green (Biolab, USA), followed by agarose gel electrophoresis. The agarose gel was 1.0% (w/v) with 0.5 X Tris Borate EDTA serving as the buffer for 30 minutes at 80 volts (Franco-Duarte *et al.*, 2019). Visualization of the gel was done by using an Ultra Violet trans-illuminator (*Seriennumber: UV 31208/070200004*). A 1kbp DNA (1000 base pairs) ladder was used to estimate the band's molecular sizes. After that, a digital camera was used to photograph the gel image.

3.8.3.4 DNA Sequencing of the 16S rRNA Gene

The PCR products were purified using the QIAquick PCR purification kit by strictly following the instructions of the manufacturer (Qiagen, Tiangen, China). In the PCR reaction, five picomoles of the forward and reverse primers were used in a 20 µl column with a DNA template of 2.5 µl. After PCR product purification, there was 500bp sequencing (Johnson *et al.*, 2019), which was carried out at Macrogen Europe Laboratory in Amsterdam-Netherlands, where the Sanger sequencing method was used, which utilized the automated sequencer ABI 3700 DNA with Big Dye Terminator Kit V. 3.1,

and the instructions of the manufacturer were followed in the reactions (Applied Biosystems, USA).

3.8.3.5 Phylogenetic Grouping Tree

The phylogenetic grouping tree was constructed using MEGAX Software. Strains were clustered together based on the similarity of sequences. Moreover, 16S rRNA gene sequences formed the basis of the Maximum Likelihood phylogenetic tree in which closest relatives' bootstrap values were calculated and indicated (1000 replications). The tree's scale was 0.05 and the same unit for branch lengths as those of evolutionary distances. The Maximum Likelihood model was used to infer the evolutionary history, based on the Tamura-Nei model. In total, there were 36 nucleotide sequences in the analysis, which led to a total of 1804 positions in the dataset.

3.9 Gas Chromatography-Mass Spectrometry Analysis of Degradation Metabolites

Four isolates with the greatest potential of biodegradation were further monitored using gas chromatography-mass spectrometry (GC-MS) to identify degradation Chlorpyrifos metabolites. Liquid samples (10 ml) were added to 50 ml of deionized water and extracted using 20 ml hexane on a rotary shaker for one hour (Shah et al., 2021). Dehydration of extracts was carried out using anhydrous Na_2SO_4 followed by drying by evaporation under Nitrogen at 45 °C in a rotary evaporator. Afterward, there was dilution with acetone to a final volume of 10 ml for analysis by chromatography. Samples were analyzed using Agilent 7890 A gas chromatography (Agilent Technologies, Inc., Beijing, China) and a 5975 C inert mass detector under the following conditions: Inlet temperature of 270 °C, with 280 °C transfer line temperature, and the column oven temperature programmed from 35 to 280 °C, with the initial maintained for five minutes followed by

an increase of 10°C/minute for 10.5 minutes while the final one was 30 minutes of 50°C/minute to 285 °C. The carrier gas used was Helium, which was flowing at 1.25ml/minute. Moreover, the electron impact (EI) mass spectra were obtained at an acceleration energy of 70 eV. Aliquots of 1.0 µL sample extract were injected directly by the auto-sampler under a quadruple technique (QMS) (Yang et al., 2019).

Fragment ions were analyzed over the 40-550m/z mass range in the full scan, with the filament delay time set at 3.3 minutes (Shah et al., 2021). The metabolic products of CP degradation were confirmed by GC-MS through the retention times of peaks. Mass spectra and the National Institute of Standards and Technology (NIST) library identification program was used to confirm the metabolites, based on the characteristic fragment ion peaks and molecular ion (m/z).

3.10 Spectrophotometric Determination of Calcium Levels

The Determination of calcium levels in milk was done using UV-VIS Spectrophotometer (AVI 2700 double beam, Thermo Fisher Scientific). The Eurochem Calcium Kit was used for the quantification. The principle of the kit is that calcium in an alkaline medium combines with O-Cresophthalein Complexone to form a purple-colored complex (Calcium + OCPC = Purple colored complex) (Gitelman (2007)). The intensity of the color formed is directly proportional to the amount of calcium present in the sample. The milk samples were first diluted by a factor of 10. The diluted samples were then centrifuged at 2000rpm for 5 minutes. The obtained supernatant was transferred to clean test tubes. After the mixing, incubation was done for 5 minutes at room temperature. Afterward, absorbance readings were done within 60 seconds, with test sample and

standard compared with the blank. The following formula was used to determine the calcium concentration in the sample.

$$\text{Calcium Concentration} = \frac{\text{Absorbance of the sample} \times 10 \frac{\text{mg}}{\text{dl}} \times 10}{\text{Absorbance of the standard}}$$

3.11 Data Management and Analysis

Data generated from the questionnaire was analyzed for descriptive statistics. The data on organophosphate levels, calcium levels, and CFUs were analyzed for inferential statistics for analysis of variance (ANOVA), t-test, and Pearson correlation analysis using SPSS software (version 20), and means were separated by Tukey's honest significant difference (HSD) test. For molecular analysis, the BLAST search program was used to compare the sequences with those in public databases on the National Center for Biotechnology Information (NCBI). Sequence similarity matrices and a phylogenetic tree were calculated and visualized using the Tree View of the MEGAX software (Kumar *et al.*, 2018). The sequences were deposited in the NCBI GenBank.

CHAPTER FOUR

RESULTS

4.1 Pre-Study Survey

The survey revealed that all the interviewed dairy farmers in Nakuru County use acaricides regularly to prevent and control ticks in cattle and other livestock. The frequency of acaricide used in dairy farms in Nakuru County revealed the use of eight different formulations. The main acaricides used are Duodip, Triatix, Ectomin, Grenade, Diazinon, Delete, and Tixfix (Figure 4.1). Of all the acaricides, Duodip was the most used at 36 %. Duodip is an acaricide combining the organophosphate Chloropyrifos with pyrethrin and pyrethroid. It is the most commonly used chemical for tick control in the farms surveyed. Triatix was the second, at 16%. Triatix contains Amitraz (a triazapentadiene). Grenade and Ectomin are synthetic pyrethroids, while Tixfix is a benzoylphenyl urea. Another OP acaricide used, though not commonly, is Diazinon. Since the proposed study focuses on organophosphates, only farms using acaricides based on organic chemicals were selected for sample collection.

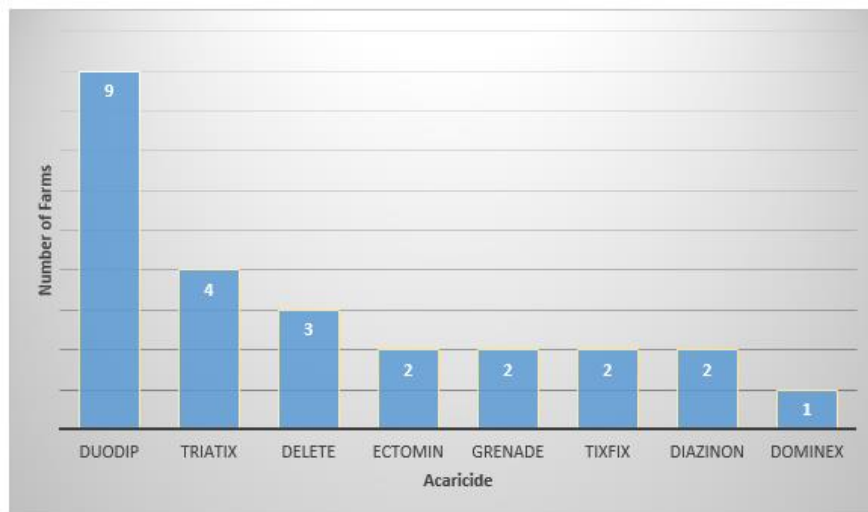


Figure 4.1: Different acaricides used by farmers in Nakuru County

The survey also observed that the main methods used for applying acaricides were dip baths, spray races, and mechanical (hand) sprays (Table 4.1). Besides, it was observed that proper drainage system lacks in most farms, with the residues ending up in agricultural farms and water sources for humans and animals. The run-off from acaricide applications are often left to freely drain into the soil for majority of the farmers, while there was pit drainage for some. Nine farmers applied modern methods of disposal, including septic tanks and pressure pumps.

Table 4.1: Method and Frequency of Acaricide Application by Farmers in Nakuru County

Parameter	Number of Farmers	Percentage (%)
Method of Application		
Dipping and spray race	6	18.75
Dipping	6	18.75
Spray race	14	43.75
Hand or mechanical spraying	6	18.75
Total	32	100.00
Frequency of Spraying		
Weekly	18	56.25
Twice weekly	7	21.88
Thrice weekly	4	12.5
Once monthly	1	3.13
Twice monthly	1	3.13
Thrice monthly	1	3.13
Total	32	100.00

Besides, the survey revealed that majority of the farmers do not milk their cows close to the places of spraying (Table 4.2). The most preferred methods for milking by the farmers is the use of automatic machines (90.62 %). Concerning the method of grazing, majority of the farmers (62.75 %) preferred fenced pastures. Other methods used were zero grazing and free range.

Table 4.2: Place, method of milking, and method of grazing used by farmers in Nakuru County

Parameter	Number of Farmers	Percentage (%)
Separation of Spraying and Milking		
Milking place close to place of spraying	8	40
Milking place distant from place of spraying	24	60
Method of Milking		
Manual	3	9.38
Automated Machines	29	90.62
Grazing method		
Zero Grazing	5	15.63
Free range	7	21.8
Fenced Pastures	20	62.5
Total	32	100.00

4.2 Presence of Chlorpyrifos in the samples

The HPLC analysis of milk samples from Nakuru County showed that many had significant levels of Chlorpyrifos levels. A Chromatogram for positive sample is shown in Figure 4.4.

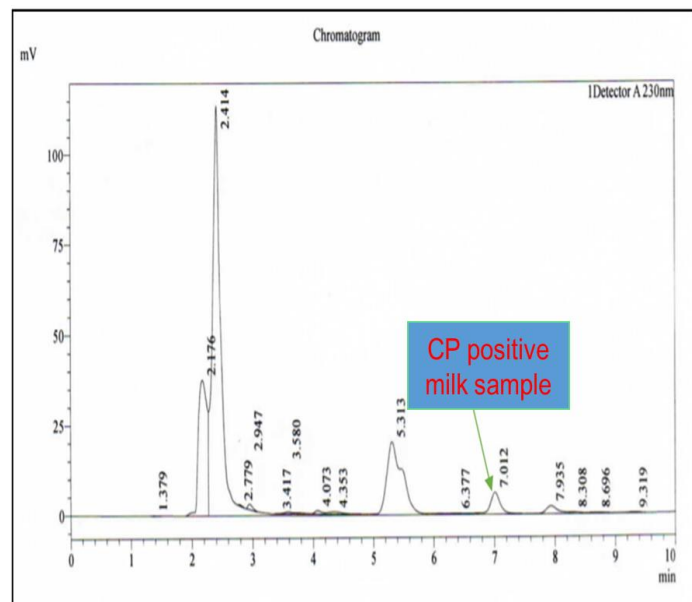


Figure 4.2: Chromatogram of a CP-positive milk sample

The HPLC analysis identified CP residues in some Soil, Water, and Dip wash samples (Table 4.3). Chlorpyrifos residues were found in raw milk samples from eight (8) out of the 15 farms, which represents 53.3% of the samples. Residues were detected in raw milk samples from all the sub-counties: two farms each for Njoro, Rongai, and Naivasha, and one farm each for Molo and Subukia. Thus, it was determined that there is no significant difference in CP contamination across the sub counties in Nakuru County ($p > 0.05$). No residues were detected in processed fresh milk or high value milk products. A t-test showed that the mean CP residues in milk from Nakuru county was significantly higher than the acceptable daily intake (ADI) of 0.01mg/litre ($p < 0.01$). Moreover, eight soil samples, five dipwash samples, and three water samples were contaminated. One way ANOVA revealed that the differences in mean concentration for milk, water, dipwash, and soil were not significant ($p = 0.099$).

Table 4.3: HPLC analysis results for presence and concentration of CP in samples

Mean CP in samples (mg/l) and SD (n = 3)					
Farm Code	Sub-county	Milk	Soil	Water	Dipwash
NF1	Njoro	2.623 ± 0.738	1.142 ± 0.281	BDL	3.354 ± 0.546
NF2	Njoro	2.721 ± 0.702	2.166 ± 0.20	1.898 ± 0.273	1.37 ± 0.106
NF3	Njoro	BDL	BDL	BDL	0.516 ± 0.048
NF4	Rongai	2.163 ± 1.031	BDL	1.793 ± 0.45	0.855 ± 0.045
NF5	Rongai	BDL	1.526 ± 0.404	BDL	BDL
NF6	Rongai	0.9275 ± 1.30	BDL	BDL	BDL
NF7	Subukia	1.518 ± 1.031	1.065 ± 0.280	BDL	BDL
NF8	Molo	BDL	BDL	BDL	BDL
NF9	Subukia	BDL	9.627 ± 0.242	BDL	BDL
NF10	Molo	BDL	BDL	BDL	BDL
NF11	Molo	2.707 ± 0.351	1.337 ± 0.149	1.777 ± 0.58	11.92 ± 2.43
NF12	Subukia	BDL	BDL	BDL	BDL
NF13	Naivasha	1.194 ± 1.052	1.612 ± 0.70	BDL	BDL
NF14	Naivasha	1.528 ± 0.875	1.679 ± 0.053	BDL	BDL
NF15	Naivasha	BDL	BDL	BDL	BDL
Mean		1.923	2.52	1.82	3.603
P values		>0.05	>0.05	>0.05	>0.05

Key. NF means Nakuru Farmer and BDL means Below Detection Limit

4.3 Estimation of Bacterial Population in Raw Milk

Table 4.4 shows the mean CFUs per sample. A one-way ANOVA revealed that some of the samples had CFUs that were significantly greater than the standard set by EU of TBC $\leq 1 \times 10^5$ CFU/ml.

Table 4.4: Mean Colony Forming Units per mL in Raw Milk Samples

Sample Code	Mean cfu/mL ($\times 10^5$)	Sample Code	Mean cfu/mL ($\times 10^5$)
NF1S1	3.0	NF7S1	520
NF2S1	51.0	NF8S1p	140
NF3S1	4.0	NF8S1Q	340
NF4S1	330	NF9S1	4.1
NF5S1	18.0	NF10S1	7.7
NF6S1	5.0	NF5S1b	18.0

The results of a Pearson Correlation analysis indicate that there is a weak, insignificant positive correlation between CFUs and Chlorpyrifos concentration in positive milk samples ($r = 0.019$, $p = 0.952$).

4.4 Soil Physical-Chemical Characteristics

The soil analysis shows that the pH of the soil from region N1 (Subukia) was medium alkaline, while the one from region N2 and N3 (Nakuru Central and Molo, respectively) were near neutral. The total nitrogen and carbon levels across all sites were adequate. Potassium, Calcium, and Magnesium levels were high in all the soils, while Sodium was adequate (Appendix 6).

4.5 Isolation of Chlorpyrifos degrading Bacteria

4.5.1 Growth in Minimum Salt Medium

Figure 4.3 presents the growth curve of bacteria in MSM in the three different concentrations as measured through optical density using UV-VIS Spectrophotometry.

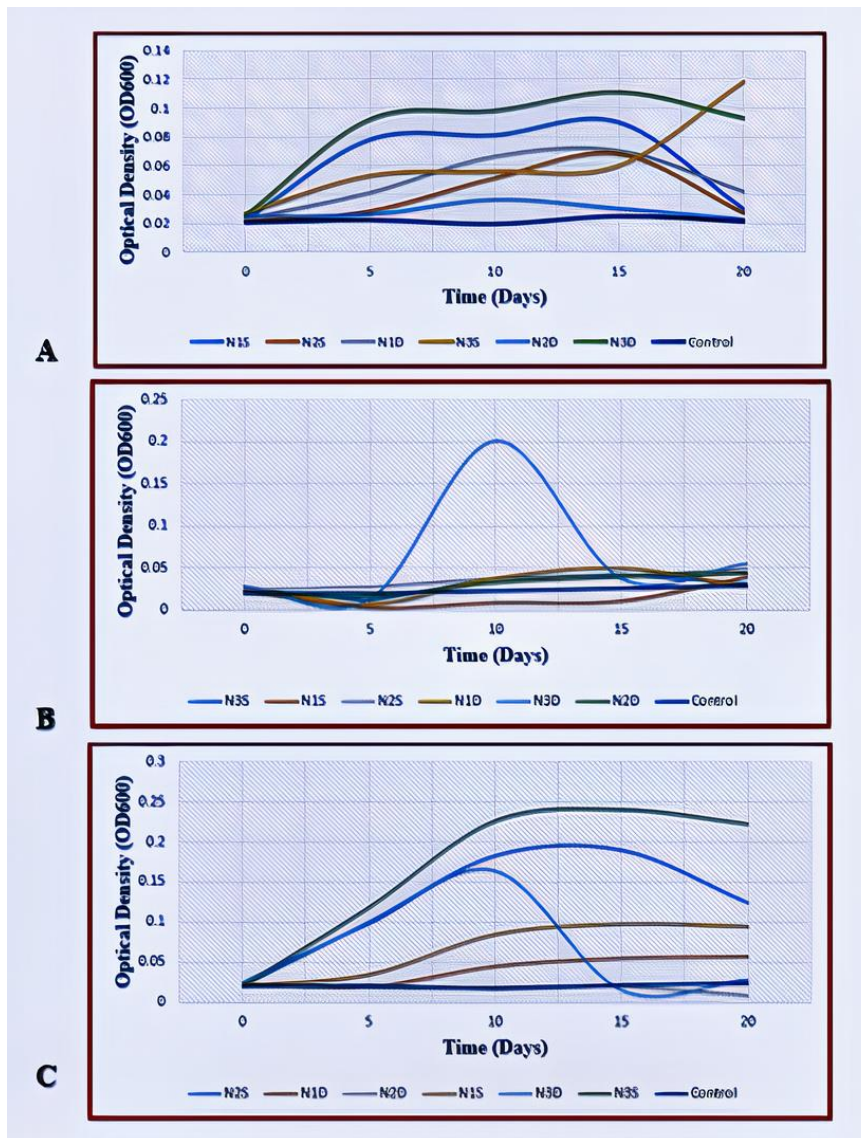


Figure 4.3: Microbial growth curves of chlorpyrifos-enriched MSM cultures (A- 5 ppm; B- 10 ppm; and C- 40 ppm). Abbreviations: N1S- Nakuru soil 1; N2S- Nakuru soil 2; N3S- Nakuru soil 3; N1D- Nakuru Dip wash 1; N2D- Nakuru Dip wash 2; and N3D- Nakuru Dip wash 3. Control- Uninoculated CP-supplemented MSM media.

There was no statistically significant difference in growth on the three different concentrations ($p = 0.99$). Pure colonies were obtained through subsequent sub-culturing in NA media (Plate 4.1).

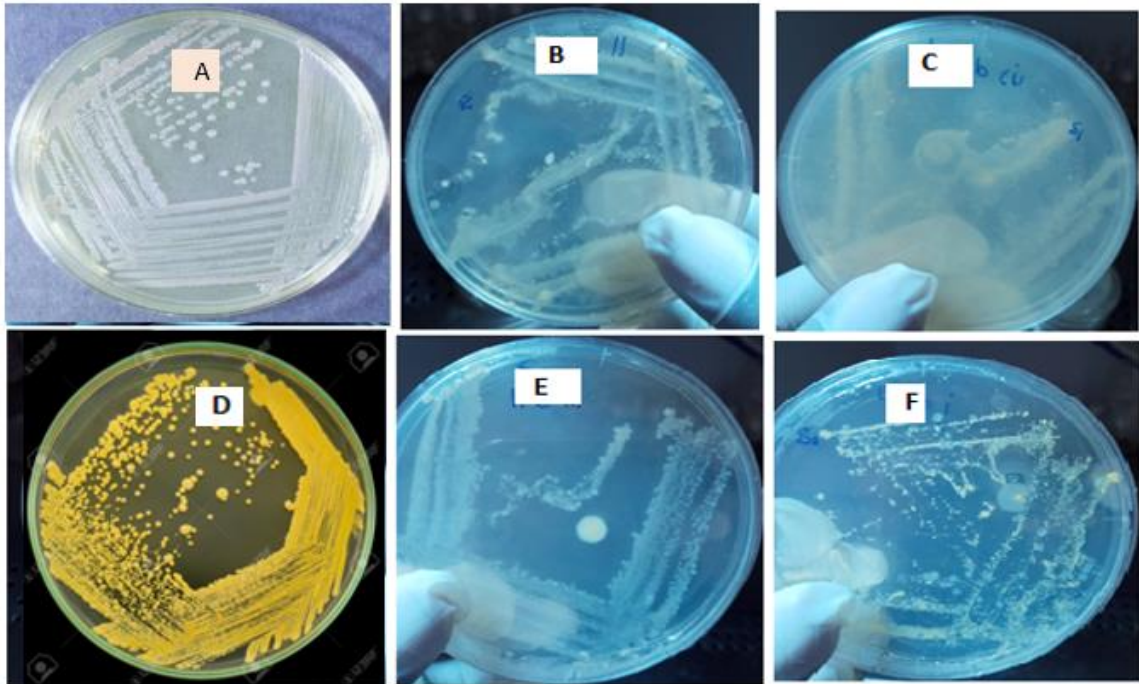


Plate 4.1: Pure Isolates of CP-degrading Bacteria grown on Nutrient Agar. A is *Alcaligenes faecalis*, B is *Lysinibacillus sp.*, C is *Pseudomonas Protegens*, D is *Achromobacter insuavis*, E is *Stenotrophomonas maltophilia*, and F is *Pseudomonas putida*.

4.6 Morphological and Biochemical Reactions of the Isolates

The 18 isolated pure colonies of potential CP degrading bacteria were designated using the codes MA1 to MA18. Under the microscope, all the isolates were rod-shaped, 4 of them (22.2%) gram-positive (MA1, MA6, MA10, and MA16), and the rest were gram-negative. Isolates MA1 and MA8 were catalase-negative, while the other sixteen were positive. Results for the Oxidase test revealed that all the isolates were positive. None of the isolates was Urease or Methyl-red positive, while only one was Indole positive

(MA1) and five lactose positive (MA1, MA13, MA16, MA17, and MA18). Only isolate MA10 and MA16 were Citrate positive, and Hydrogen Sulfide production was only evident in isolate MA1 and MA16.

4.7 Molecular Characterization of Bacterial Isolates

4.7.1. PCR Amplification of isolates

All the 18 isolates were shown to have PCR amplified fragments with around 1500bp DNA (Figure 4.4).

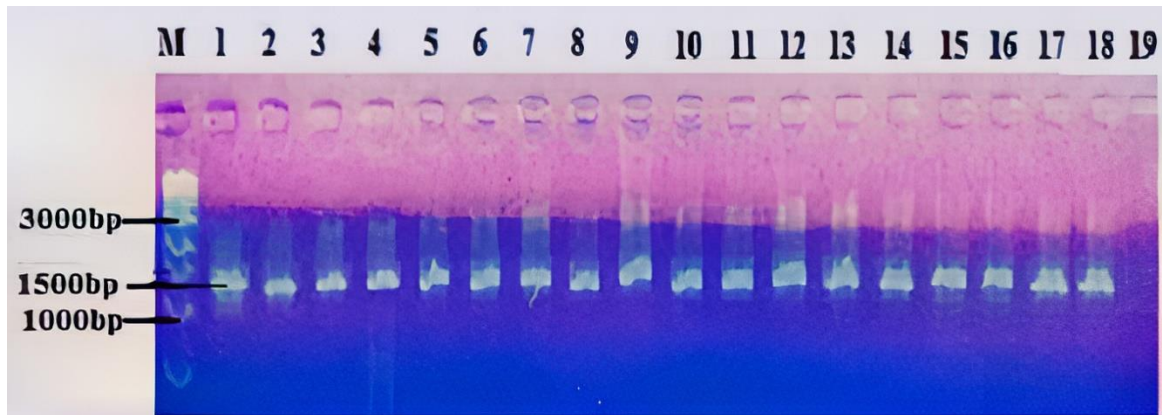


Figure 4.4. Agarose Gel Image of PCR amplified 16S rDNA of the bacteria isolates Lane M, 1kb DNA ladder (Biolabs); Lanes 1 – 18, 16 S rDNA of bacteria isolates (MA1 - MA18); Lane 19, Negative control.

4.7.2. 16S rRNA Sequence Analyses.

Following NCBI GenBank blasting of the 16S rRNA sequences of the bacteria isolates, nine of these isolates (MA3, MA4, MA5, MA7, MA9, MA11, MA12, MA14, MA15) belonged to the genus *Pseudomonas*, three (MA13, MA17, MA18) belonged to the genus *Alcaligenes*, three (MA1, MA6, MA16) to the genus *Bacillus* and one (MA15) belonged to *Achromobacter* (Table 4.5).

Table 4.5: Identify of isolated bacteria based on the 16S rDNA sequence

Isolate	Accession	Species (16S rRNA gene analysis)	Accession	Identity (%)	Sequence length (bp)
MA1	MZ314427.1	<i>Lysinibacillus sp. HBUM206408</i>	MT541001.1	100	1201
MA2	MZ359883.1	<i>Stenotrophomonas maltophilia B9</i>	JQ900524.1	96.29	1038
MA3	MZ310718.1	<i>Pseudomonas protegens L21</i>	MT505104.1	96.4	1418
MA4	MZ310719.1	<i>Pseudomonas putida TCA1</i>	JQ782512.1	100	1463
MA5	MZ359884.1	<i>Pseudomonas putida strain c275</i>	JQ782512.1	96.35	1039
MA6	MZ310720.1	<i>Bacillus sp. C4P019a</i>	FJ950565.1	96.56	1451
MA7	MZ310721.1	<i>Pseudomonas sp. IAE245</i>	MN989109.1	92.9	1423
MA8	MZ314428.1	<i>Achromobacter insuavis</i>	MK414951.1	90.81	1277
MA9	MZ359885.1	<i>Pseudomonas fluorescens strain psf4</i>	MF662233.1	98	1420
MA10	MZ359886.1	<i>Brevundemonas aurantiaca ZJY-891</i>	MN256390.1	89.53	1417
MA11	MZ310722.1	<i>Pseudomonas vranovensis S3-4</i>	GU201990.1	98.8	1411
MA12	MZ310723.1	<i>Pseudomonas aeruginosa 31</i>	LC425424.1	98.11	1267
MA13	MZ310724.1	<i>Alcaligenes faecalis KWW 84</i>	MN732983.1	96.98	1427
MA14	MZ310725.1	<i>Pseudomonas sp. FBF19</i>	LC425424.1	90.73	1436
MA15	MZ314429.1	<i>Pseudomonas plecoglossicida B3</i>	LK391651.1	90	1198
MA16	MZ310726.1	<i>Bacillus sp. FJAT-22078</i>	HG805697.1	93.54	1405
MA17	MZ310727.1	<i>Alcaligenes faecalis H11</i>	MT071410.1	98.15	1460
MA18	MZ310728.1	<i>Alcaligenes sp. A23</i>	KY949530.1	96.38	1272

The phylogenetic grouping tree is presented in Figure 4.5. Reference type strains of corresponding bacteria are involved in the tree.

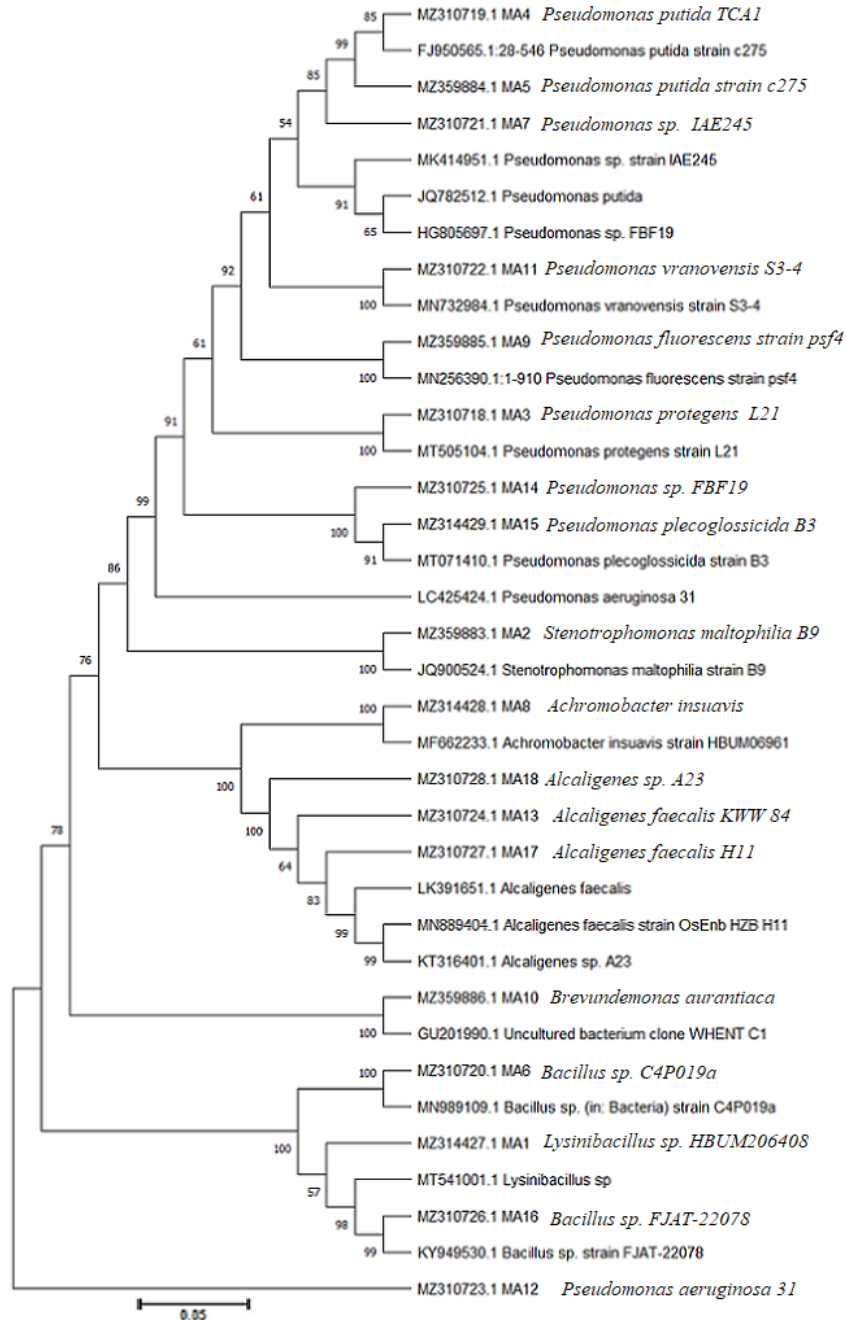


Figure 4.5: Phylogenetic tree showing the relationship of the isolates to closely related bacteria

4.8 GC-MS Analysis

Two peaks were observed at retention time of 13.40 minutes and 18.87 minutes, corresponding to the chlorpyrifos standard and the TCP metabolite. The sample had a m/z value of 350.76 on day zero, confirming the presence of chlorpyrifos in the medium. TCP was produced as the breakdown metabolite for all strains after incubation, with a m/z value of 197. The chlorpyrifos peak disappeared concomitantly with the production of the TCP peak. The retention time of CP and TCP is presented in Figure 4.6 while the chemical structure and mass spectra is given in Figure 4.7.

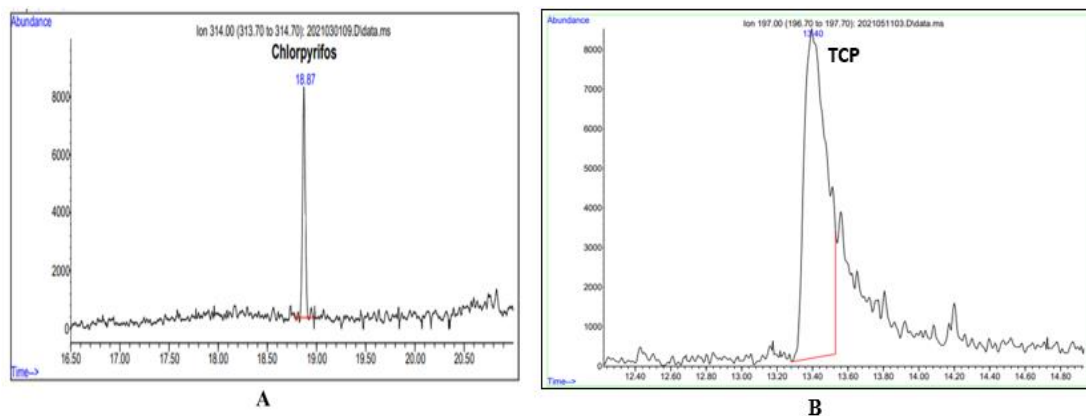


Figure 4.6: GC chromatograms of CP and TCP: Retention time of CP at 18.87 minutes (A) and TCP 13.40 minutes (B) and other smaller unidentified metabolites.

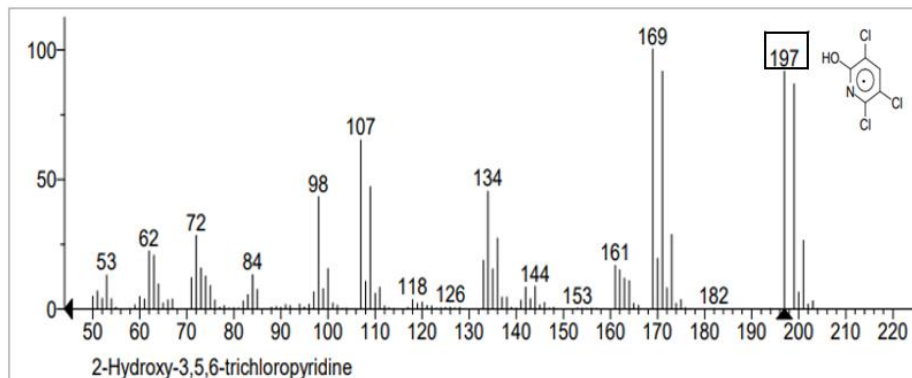


Figure 4.7: Chemical structure and mass spectra of TCP produced from chlorpyrifos degradation

The rate of degradation of Chlorpyrifos was very low in the uninoculated control during the 12 days period of analysis since the percentage of Chlorpyrifos degraded was at the end of the period was only 8.08% (Figure 4.8). In comparison, all the four isolates showed significant degradation, that is: MA1 (*Lysinibacillus* sp.); 79.52%, MA4 (*Pseudomonas putida*); 73.32%, MA2 (*Stenotrophomonas maltophilia*); 71.43%, and MA8 (*Achromobacter insuavis*) 71.03%. A two-way ANOVA found no significant difference in the rate of CP degradation by the four isolates ($p = 0.442$).

Table 4.6: Chlorpyrifos biodegradation in MSM medium (initial concentration of 25 mg/l, temperature at 30°C) for a period of 12 days

		Incubation Time (Days)				
		Day Zero	Day 4	Day 8	Day 12	Day 16
Control	Concentration	25	24.76±0.07	23.61±0.54	22.98±0.66	22.11±0.06
	%	0	0.96	5.56	8.08	11.56
MA1	Conc	25	14.64±0.69	11.06±0.93	5.12±0.19	3.21±0.11
	%	0	41.44	55.76	79.52	87.16
MA2	Conc.	25	10.7±0.08	7.62±0.05	7.14±0.06	4.49±0.06
	%	0	47.67	69.52	71.43	82.04
MA4	Conc.	25	17.65±0.21	9.86±0.06	6.67±0.04	2.62±0.07
	%	0	29.4	60.56	73.32	89.52
MA8	Conc	25	12.16±0.49	8.67±0.47	3.69±0.62	2.23±0.06
	%	0	42.8	54.43	71.03	91.08

A two-way ANOVA found no significant difference in the rate of CP degradation by the four isolates ($p = 0.442$), but there was a significant difference between the isolates and the control ($p < 0.05$).

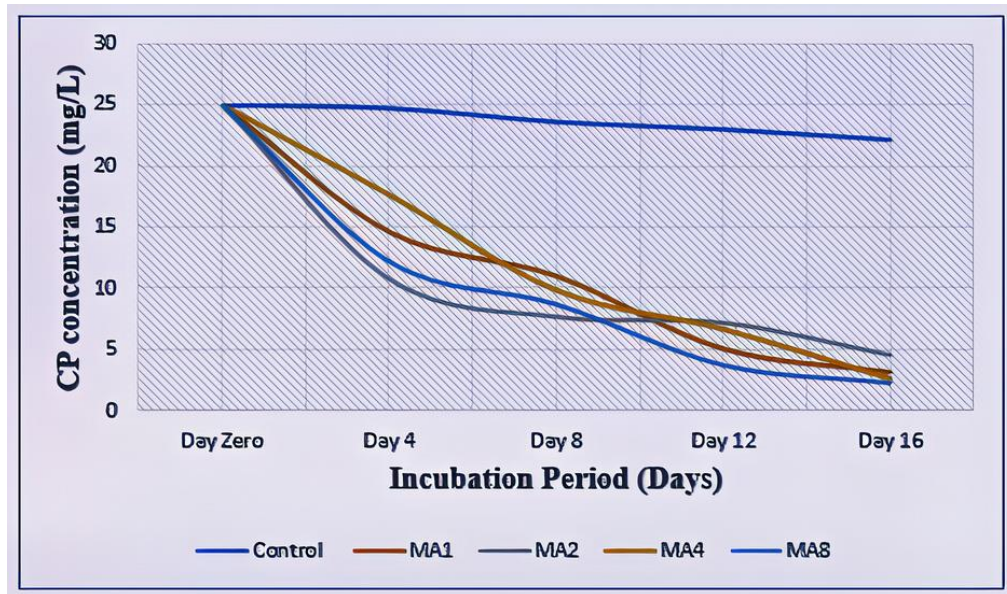


Figure 4.8: Degradation kinetics of CP by four bacterial isolates and the control

4.9 Calcium Concentration in Milk and Milk Products from Nakuru County

Table 4.7 gives calcium concentration in different milk samples from Nakuru County. Reference levels for calcium concentration is 1209 mg/L (Vyas and Tong, 2003). Most of the milk samples had calcium concentrations within the normal range of between 120-250 mg/dl. The mean calcium concentration of the samples was 279.26, which is not significantly different from the normal range. One sample of raw milk had very low levels of calcium (85.73 mg/dl), and three had extremely high concentrations, such as MOLOS1 (603.74 mg/dl), MP6 (513.89 mg/dl), and NF5S1 (544.54 mg/dl). No significant difference was observed between calcium concentrations in raw milk and processed milk products.

Table 4.7: Calcium Concentration in milk samples from Nakuru County

Sample Code	Type of Sample	Mean Calcium Concentration (mg/dl)
NF1S1	Raw milk	198.28 ± 7.02
NF2S1	Raw milk	264.66 ± 14.20
NF3S1	Raw milk	295.16 ± 12.00
NF4S1	Raw milk	231.90 ± 13.43
NF5S1	Raw milk	544.54 ± 12.02
NF6S1	Raw milk	197.97 ± 6.86
NF7S1	Raw milk	346.17 ± 4.93
NF8S1P	Raw milk	231.66 ± 8.82
NF8S1Q	Raw milk	85.72 ± 7.11
NF9S1	Raw milk	124.80 ± 7.21
NF10S1	Raw milk	192.12 ± 12.70
EUY	Yoghurt	262.24 ± 7.67
MOLOS1	Raw milk	603.74 ± 5.62
MOLOYG	Yoghurt	215.99 ± 8.00
NF5S1B	Malai	343.06 ± 5.65
MP4	Pasteurized milk	202.63 ± 6.82
MP6	Yoghurt	513.89 ± 9.00
MP2	Cheese	237.11 ± 5.05
MP5	Butter	202.95 ± 6.03
MP3	Pasteurized milk	299.28 ± 5.95
MP1	Yoghurt	270.55 ± 14.00
Mean		279.26
P value		>0.05

A Pearson correlation analysis showed that there was a weak, negative relationship between Chlorpyrifos levels and calcium concentration in milk ($r = -0.2$, $p = 0.636$). Therefore, it was affirmed that the effect Chlorpyrifos residues on the concentration of Calcium in milk was not significant. A scatter plot showing the relationship between CP and calcium is presented in Figure 4.9.

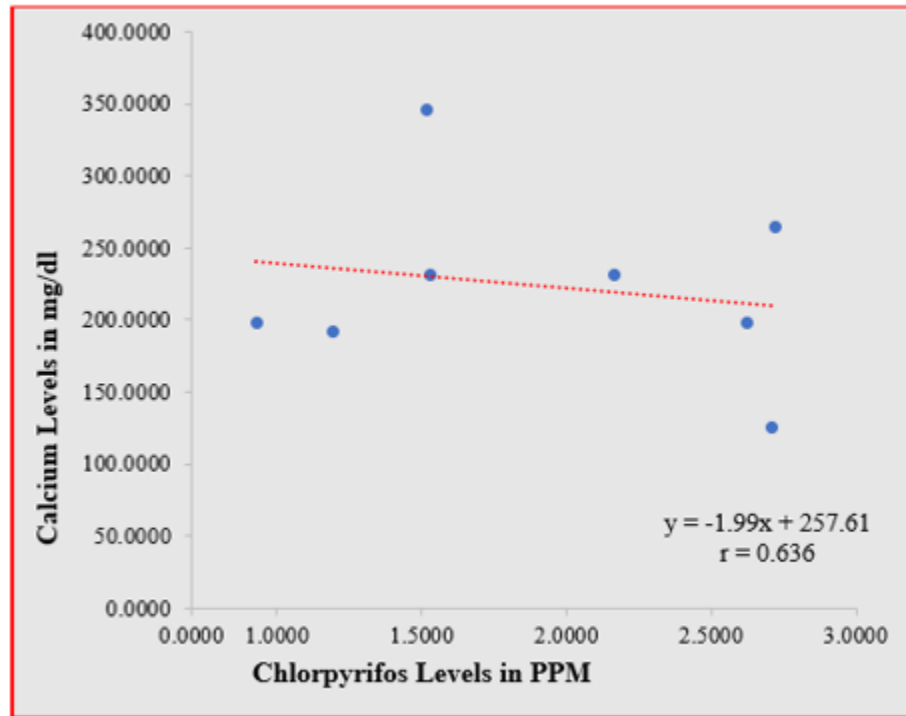


Figure 4.9: Pearson Correlation Coefficient scatter plot showing line of best fit for correlation between Chlorpyrifos and calcium

CHAPTER FIVE

DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Use of Organophosphates as Acaricides in Nakuru County

According to the findings of the pre-study survey many farmers in Nakuru County were found to use acaricides to control ticks. Among the most commonly used are the organophosphates, especially Chlorpyrifos. The findings are consistent with literature that shows that organophosphates are the most applied insecticides, accounting for around 38% of the pesticides used globally (Abdel-Wareth and Abd El-Hamid, 2016). Intensive use of these chemicals causes contamination of soil, water, and air (Jayasinghe *et al.*, 2019). Residues of the chemicals can be absorbed by dairy animals through contaminated air, water, and feed. CP can persist in water for up to 78 days and in the soil for up to 120 days (Chishti *et al.*, 2013). However, farmers in the study region are compelled to use acaricides to control ticks, which are a persistent menace in the Kenyan Rift Valley, where they are responsible for various diseases, including the East Coast Fever (Beckley *et al.*, 2016; Mutavi *et al.*, 2018).

5.2 Organophosphate residue levels in Samples from Nakuru County

The study was among the first research to analyze Chlorpyrifos residue levels in milk, milk products, soil, water, and dip wash Nakuru County, Kenya. Results of this study show that there are significant levels of Chlorpyrifos residues in raw milk from Nakuru County, which makes it unsafe for human consumption. The findings corroborate the study by Abdel-Wareth and Abd El-Hamid (2016), which reported that only a small percentage of applied pesticide is actually beneficial in reaching their target and that majority of it is dispersed through the environment.

Based on the findings of this study, it was determined that there was contamination of milk samples with organophosphates in all the five sub-counties that were analyzed. The results show that contamination of raw milk is widespread in Nakuru County, thus pointing to the fact that majority of the raw milk samples from selected farms in the region may not be fit for human consumption. According to Aslam *et al.* (2013), milk is the most versatile organic food that is obtained from animals. The presence of OP residues in milk affects the quality and safety of milk that the animal produces. Consequently, it poses a health risk to consumers, who are exposed to toxic organophosphate residues.

The levels of CP in milk samples were higher than the maximum residue limits of 0.01 mg/l as recommended by WHO. The findings are consistent with previous studies, which have shown that there is prevalent CP contamination in dairy milk (Aslam *et al.*, 2013; Deti *et al.*, 2014; Bedi *et al.*, 2015; Jayasinghe *et al.*, 2019). Locally, the findings corroborate the study by Miriti *et al.* (2014) and Anode *et al.* (2018), which reported significant levels of organochlorides and organophosphates in soil samples from Nakuru County.

However, the present study did not find detectable levels of CP in selected high-value milk products (pasteurized, fermented, sterilized, and milk powder). Notably, value-added milk products show lower pesticide residues compared to fresh milk. This occurrence could be because processes used in the development of high-value products, including pasteurization and homogenization, lead to the elimination of OP residues

(Jayasinghe *et al.*, 2019). Moreover, the half-life of OP acaricides in the environment is relatively short, which explains why they occur in raw milk but not in preserved long-life milk products.

The source of organophosphate contaminants for the milk could most probably be the feed used by farmers (Jayasinghe *et al.*, 2019). In Nakuru, most dairy farmers feed their cattle with crop residues, grasses, and animals, which could be contaminated by either acaricide used for tick control or herbicides that are extensively applied in the area. Grass and feed given to the cattle could be contaminated, and after feeding, they pass through the body systems (Jayasinghe *et al.*, 2019). Other factors that contribute to contamination include the mode of application of acaricides, the type of acaricide used, frequency of application, methods of disposal, and accidental spills. The study also found out that soil and water samples from dairy farms that were selected from different regions in Nakuru County are contaminated with organophosphates.

5.3 Total Bacteria Population in Milk in Nakuru County

Although milk is virtually a sterile fluid when in the alveoli of the udder, microbial contamination occurs from different sources (Pereira *et al.*, 2009; Daka *et al.*, 2012; Pysz-Lukasik *et al.*, 2015; Tóth *et al.*, 2020). The findings of this study show that the freshly collected raw milk samples from Nakuru County had significant CFUs, which are beyond the recommended limit. According to Reta *et al.* (2016), microbes prefer milk because it is rich in nutritional content. Therefore, care is needed in handling milk to avoid microbial contamination. Notably, the study did not find a significant correlation between OP residue levels and CFUs in milk.

5.4 Isolation of Organophosphate degrading Bacteria

In this study, the isolated bacteria belong to the genera of *Pseudomonas*, *Stenotrophomonas*, *Alcaligenes*, *Bacillus*, and *Achromobacter*. Members of these genera have been isolated before with the ability to degrade a variety of chemical compounds in the environment (Abo-Amer, 2011; Ambreen and Yasmin, 2021). Microbial degradation is a significant and efficient method for eliminating harmful compounds from the environment (Abdel-Wareth and Abd El-Hamid, 2016). Biodegradation of OPs in the soil and water is important because of the potential of these chemicals to get into the livestock and eventually into human consumers of animal products (Mie et al., 2018). According to Yadav *et al.* (2015), bacteria have the ability to degrade pollutants efficiently because they have catabolic genes that enable them to survive in different ecological niches in various pH, temperature, and oxygen concentrations.

Various previous studies have isolated bacterial communities with the potential to degrade CP and TCP in the soil and in liquid culture. They include *Arthrobacter sp.*, *Enterobacter strain B-14*, *Alcaligenes faecalis*, *Bacillus pumilus* and *Pseudomonas aeruginosa* (Singh *et al.*, 2004, Anwar *et al.*, 2009, Yadav *et al.*, 2015). These bacterial communities have been shown to catabolize and co-metabolize TCP and CP. Yadav *et al.* (2015) reported co-metabolization of CP by *Arthrobacter sp.* and *Flavobacterium sp.* However, research has shown that the organism does not use CP as the energy source. Singh *et al.* (2004) reported that, instead, the bacterium hydrolyses CP to TCP and diethyl thiophosphate (DETP) and then uses the latter for growth and energy.

Although the majority of bacteria strains that degrade CP are limited by TCP, some species of *Pseudomonas* mineralize the compound. Moreover, some strains of *Alcaligenes* are capable of biodegrading both TCP and CP (Yadav *et al.*, 2015). In the present study, the majority of the bacterial communities isolated belong to the two genera, thereby corroborating previous findings. Nonetheless, the study also identified other genera with CP degradation ability that have not been widely documented in the literature, including *Lysinibacillus fusiformis*, *Stenotrophomonas maltophilia*, and *Achromobacter insuavis*.

GC-MS analysis showed that the four bacterial isolates (MA1, MA2, MA4, and MA8) effectively degraded Chlorpyrifos to produce TCP. They were able to remove between 82 % and 91 % of the initial 25 mg/l of CP in MSM media without an additional source of carbon in 12 days, which shows that these isolates are able to degrade the acaricide without additional nutrients such as glucose for inducing OP degradation enzymes. These findings are consistent with previous studies that have shown the main product of degradation to be TCP (Chen *et al.*, 2012). Some studies have reported almost 100% degradation of CP in MSM broth (Ambreen *et al.*, 2020). The study by Khalid *et al.* (2016) showed that, in the presence of glucose, *Pseudomonas kilonensis* SRK1 degraded CP by 50% within 24 hours.

The significant difference in degradation kinetics between the isolates and the uninoculated control show that the bacteria are efficient in bioremediation of CP residues. The degradation that occurred in the control was due to other degradation mechanisms, which include photolysis and hydrolysis. However, abiotic hydrolysis does not play a

substantial impact on chlorpyrifos degradation. Chlorpyrifos hydrolysis has been proven to be stable in neutral to acid conditions (half-life values 72 to 81 days). Nevertheless, laboratory tests suggest chlorpyrifos is vulnerable to hydrolysis under alkaline conditions (pH 9), with a half-life of roughly two weeks. TCP is the most stable hydrolysis product

In the current study, the ability of the isolates to break down CP into TCP was confirmed. Therefore, the study reports success in the use of bacteria for bioremediation of CP, corroborating previous studies that have been done elsewhere (Chen *et al.*, 2012; Abraham and Silambarasa, 2013). It is important to emphasize that the laboratory conditions are normally used as model ecosystems to mimic the natural environment, with controlled conditions such as pH, temperature, humidity, and light. The use of a control experiment in the current study helped to monitor physical degradation through abiotic methods. The degradation rate in the control setup, which did not include any microorganism, was significantly slower than biodegradation therefore, it confirms that the isolated microorganisms contribute to enhanced degradation. Ester hydrolysis was the primary pathway for chlorpyrifos degradation, leading to production of TCP.

The bacterial consortium isolated from the samples contained a group of strains with the ability to degrade the organophosphate pesticide significantly. However, it is important to note that the existing laboratory methods can only isolate around 10% of all bacteria. Therefore, many of the bacteria that interfere with the degradation process may not be isolated or cultured in the laboratory (Ortiz-Hernández and Sánchez-Salinas, 2010). Successful detoxification of pesticides and other organic chemicals in the soil often requires the concerted efforts of bacteria consortia (Akhdiya *et al.*, 2020).

5.5 Effect of Organophosphate Residues on Calcium Concentration in Milk

This study focused on calcium because it is one of the most abundant minerals in milk and forms complexed compounds with phosphates. To the best of my knowledge, this is the first study that has explored the effect of organophosphate pesticides on the levels of calcium in cow milk. In the normal blood plasma of cows, the Ca concentration is tightly regulated between 8.5 to 10 mg/dl (2.1 to 2.5 mM) (Wynn *et al.*, 2015). In cow milk, normal calcium levels range from 120 to 250 mg/dl. The current study found that most of the milk samples that were selected had calcium levels within the normal range, although there were some that were significantly higher or lower. A correlation analysis that compared organophosphate levels and calcium concentration only found a weak, insignificant negative relationship. The findings of the present study, therefore, cannot be used to conclude that presence of organophosphate metabolites interfere with calcium concentration in milk.

A lot of focus has been put on calcium because many adults do not consume the recommended quantity of the nutrient, leading to calcium deficiency that contributes to the development of disorders such as osteoporosis. Cow milk is the most important source of calcium for human consumption (Pšenková *et al.*, 2020). Previous studies had shown that the composition of milk, including calcium levels, can be affected by various factors, including the type of food, pre-partum nutrition, the environment, and breed (McGrath *et al.*, 2015; Linn, 2016; Albani *et al.*, 2019). According to Linn (2016), diets that reduce the fat composition of milk also lower soluble calcium in milk. Zwierzchowski and Ametaj (2019) had also reported that environmental pollution, agricultural and urban emissions could also affect milk composition. The present study

explored organophosphates as environmental pollutants because their residues persist in the environment for a long time, ending up in animal feeds. These chemicals and their metabolites accumulate in the bodies of animals, where they pass into milk and milk products.

5.6 Conclusions

- i. Significantly high levels of organophosphate residues were found in milk, soil, and water samples from Nakuru County, Kenya. Fresh milk from eight out of fifteen sampled farms in Nakuru County had Chlorpyrifos residues significantly higher than the maximum residue levels in milk products.
- ii. The present study characterized 18 bacterial strains isolated from soil and dip-wash from Nakuru County, with the potential to degrade some Organophosphate acaricides. Some of the isolates were novel Chlorpyrifos -degrading strains, and others have been reported in previous studies to degrade the organophosphate. All the isolated strains grew in the presence of Chlorpyrifos and degraded it as a source of carbon and energy. These microorganisms can be used to form a bacterial consortium which will be used for bioremediation of organophosphate pesticides. The methods conventionally used for remediation of organophosphate contaminated areas include recycling, chemical treatment, incineration, pyrolysis, and landfills, which are all not only costly but also less efficient as they can lead to the formation of toxic intermediates. Moreover, the isolated strains can be added to soil to improve quality by degrading pesticides, thereby promoting sustainable agriculture and the environment.
- iii. The study showed that the presence of organophosphate residues in milk does not significantly affect calcium ion levels in milk ($p = 0.636$), and the correlation between the two variables was weak and negative ($r = - 0.2$).

5.7 Recommendations

- i. Proper handling and processing of milk and milk products are required to minimize contamination by acaricide residues.
- ii. The organophosphate degrading bacteria obtained in this study can be used to make a consortium that is effective for bioremediation. The isolated bacteria strains from this study can be added to soils as inoculants for bio-augmentation to improve soil quality. OP-degrading genes can be obtained from these isolates and harnessed for biotechnological applications, such as the treatment of OP poisoning in animals and humans. Compatibility tests should be carried out to determine the compatibility/incompatibility potential among the isolated OP-degrading bacteria. Moreover, there is a need for further studies to determine the possible mechanisms and other metabolites of Chlorpyrifos degradation by the isolates obtained in the study.
- iii. Further studies are required to explore the effect of organophosphate residues on other essential components of milk, including other microelements such as sodium, potassium, and magnesium.

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APPENDICES

Appendix 1: Survey Questionnaire

KENYATTA UNIVERSITY

QUESTIONNAIRE FOR NAKURU COUNTY DAIRY FARMERS' SURVEY*The information obtained will be confidential and used for research purposes only.*

Questionnaire number _____ Date _____

Name of the farm _____

Sub-County _____ Village _____

Contact details _____

SECTION A: GENERAL INFORMATIONTick where appropriate using ✓ in the box.

1. What is the type of Farm scale do you adopt?

(a) Large scale

(b) Small scale

2. What Animal breed (s) do you keep?

Number

(a) Friesian

(b) Jersey

(c) Guernsey

(d) Ayrshire

(e) Cross breed

(f) Indigenous

(g) Any other (specify).....

3. What type of animal grazing system do you use?

(a) Zero grazing unit

(b) Semi extensive

(c) Extensive grazing

(d) Any other (specify).....

4. What type of feeds do you use?

(a) Roughage

(b) Pasture

(c) Concentrate

(d) Silage

(e) Any other (specify).....

5. How many times do you feed your animals per day?

(a) Once

(b) Twice

(c) Thrice

(d) Four

6. What amount of feed do you give your animal? (per kg)

a) 20-30

b) 30-40

c) 40-60

d) 60-above

SECTION B

8. Do you source for extension services? Yes/No

If yes (explain)

.....
.....

If no explain why

.....
.....

9. Do you sell your milk fresh or processed?

a) Fresh: Yes/No

.....
If yes explain why

.....
c) Processed: Yes/No

If yes explain how you process the milk

.....
10. What is the average quantity of milk produced in liters? (per cow)

11. How do you control ticks on your farm?

a) Dipping

b) Hand spraying

c) Hand washing

d) Hand picking

e) Any other method used (specify)

12. How often do you spray your animals? (If applicable)

.....
13. What type of acaricides do you use?

.....
14. What is the disposal method of acaricides waste on your farm? (If applicable)

.....
15. What is the frequency of disposal of the contents of the cattle dip? (If applicable)

.....
16. What is the source of the water your animals drink?

Appendix 3: Kenyatta University Graduate School Research Authorization Letter



**KENYATTA UNIVERSITY
GRADUATE SCHOOL**

E-mail: dean-graduate@ku.ac.ke

Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: I56/37934/16

DATE: 4th December, 2018

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

**RE: RESEARCH AUTHORIZATION FOR MR. MICAH NYABIBA ASAMBA –
REG. NO. I56/37934/2016**

I write to introduce Mr. Micah Nyabiba Asamba who is a Postgraduate Student of this University. He is registered for M.Sc. degree programme in the Department of Microbiology, Biochemistry & Biotechnology.

Mr. Asamba intends to conduct research for a M.Sc. thesis Proposal entitled, "Microbial Degradation of Organophosphate Residues and its Effect on Calcium Levels in Fresh and Processed Milk from Nakuru County, Kenya."

Any assistance given will be highly appreciated.

Yours faithfully,



PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL


Appendix 4: NACOSTI Research Permit

THIS IS TO CERTIFY THAT: **Permit No : NACOSTI/P/19/14281/27310**
MR. MICAH NYABIBA ASAMBA **Date Of Issue : 24th January,2019**
of KENYATTA UNIVERSITY , 43844-100 **Fee Recieved :Ksh 1000**
Nairobi,has been permitted to conduct
research in Kajiado , Nakuru Counties

on the topic: MICROBIAL DEGRADATION
OF ORGANOPHOSPHATE RESIDUES AND
ITS EFFECT ON CALCIUM LEVELS IN
FRESH AND PROCESSED MILK FROM
NAKURU COUNTY, KENYA

for the period ending:
23rd January,2020

.....
Applicant's
Signature


Director General
National Commission for Science,
Technology & Innovation

Appendix 5: Approval for Use of Mount Kenya University Research Centre



OFFICE OF THE HEAD, RESEARCH CENTRE

OUR REF: MKU00/HRC/VOL1/2018/004

10th December, 2018

Mr Micah Nyabiba Asamba
Kenyatta University
P O Box 43844 - 00100
NAIROBI


Dear Mr Asamba,

**RE: APPROVAL FOR USE OF MOUNT KENYA UNIVERSITY
RESEARCH CENTRE**

Reference is made to your letter dated 31/10/2018, referenced "Request for permission to use laboratories and research facilities".

This is to inform you that permission has been approved as from the month of January to May 2019 subject to the adherence to the Research Centre Policy Guidelines

Yours sincerely,


Mount Kenya University
Head, Research Centre
P. O. Box 342 - 01003,
Thika

Dr. Jared Misonge Onyancha
HEAD, RESEARCH CENTRE
DIRECTORATE OF RESEARCH & INNOVATION

Appendix 6: Soil Analytical Data from Kenya Agricultural and Livestock Research Organization (KARLO)



Kenya Agricultural & Livestock Research Organization
National Agricultural Research Laboratories
 P. O. Box 14733, 00800 NAIROBI
 Email: soilabs@yahoo.co.uk

SOIL TEST REPORT

Name Micah Asamba
 Address
 Location of farm Nakuru
 Crop(s) to be grown
 Date sample received 18-Dec-2020
 Date sample reported 11-Jan-2021

Soil Analytical Data								
Baseline								
Field	N 1		N 2		N 3			
Lab. No/2020	7383		7384		7385			
Soil depth cm	top		top		top			
Fertility results	value	class	value	class	value	class	value	class
Soil pH	8.18	medium alkaline	6.92	near neutral	6.95	near neutral		
Total Nitrogen %	0.46	adequate	0.37	adequate	0.37	adequate		
Total Org. Carbon %	4.94	adequate	3.94	adequate	3.97	adequate		
Phosphorus ppm	104	high	145	high	90	high		
Potassium meq%	12.8	high	10.8	high	10.0	high		
Calcium meq%	16.0	high	17.2	high	19.0	high		
Magnesium meq%	6.78	high	5.82	high	5.78	high		
Manganese meq%	1.15	adequate	1.43	adequate	1.63	adequate		
Copper ppm	0.10	low	0.12	low	3.25	adequate		
Iron ppm	12.2	adequate	83.7	adequate	248	adequate		
Zinc ppm	81.8	adequate	79.8	adequate	96.2	adequate		
Sodium meq%	0.64	adequate	1.06	adequate	1.02	adequate		
Elect. Cond. mS/cm	1.25	high						

Appendix 7: Invoice for Sequencing Results from Microgen Europe B.V.



MacroGen Europe B.V.
 Meibergdreef 57
 Amsterdam 1105 BA
 Netherlands
 Company ID 69716099
 Tax ID NL857980944B01

INVOICE

Bill TO

ATTN : PAUL OSHULE SIFUNA

Mount Kenya University (NRF project),
 P.O. Box 342, 01000,
 Thika -Kenya.

VAT ID :

INVOICE#

ESC210330466

Invoice Date	Terms	Due Date	P.O.#
30-03-2021	30-03-2021	30-04-2021	

Order No.	Item	Qty	Currency	Unit Price	Amount
210312EN-013	PCR product Purification-e	25	US\$	2.5	62.5
	PCR product Sequencing	50	US\$	5	250

Sub Total	312.5
(VAT Exclusive)	
EU VAT out of scope (0%)	0
Total	312.5