BIODEGRADATION KINETICS OF CHLORPYRIFOS AND DIURON DEGRADING BACTERIAL ISOLATES FROM THE NZOIA RIVER DRAINAGE BASIN, KENYA

Eric Oliang'a Mirenga

A thesis submitted in partial fulfillment of the requirement for the award of Masters of Science degree in Molecular Biology of Masinde Muliro University of Science and Technology

DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award

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Eric Oliang'a Mirenga **Date**

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CERTIFICATION

The undersigned certify that they have read, and hereby recommend for acceptance of Masinde Muliro University of Science and Technology, a thesis entitled "**Biodegradation Kinetics of Chlorpyrifos and Diuron Degrading Bacterial Isolates from The Nzoia River Drainage Basin, Kenya"**

..... ……

Dr. Jackson Cheruiyot **Date**

Department of Biological Sciences

Masinde Muliro University of Science and Technology

..... ……

Prof. Zachary Getenga **Date**

Department of Physical Sciences

Chuka University

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DEDICATION

To God; my father Dr. Isaac Moss-Omije Mirenga, my mother Esther Mirenga, and my brother Edward Omije Mirenga.

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ABSTRACT

The pesticides Chlorpyrifos and Diuron are extensively used to control termites and perennial weeds respectively in sugarcane farming in the Nzoia Sugar Company Nucleus Estate. Biodegradation is a major avenue for removal or breakdown of these pesticides and their metabolites in soil, with the inoculum density of the biodegrading bacteria being an important factor influencing the process. In spite of the importance of this factor, there exist few studies that have extensively investigated the effects of varying inoculum density of degradative bacteria on the biodegradation of the two pesticides and their respective metabolites. The aim of this study was to isolate and characterize Chlorpyrifos and Diuron- degrading bacteria from exposed agricultural soil from the Nzoia Sugar Company Nucleus Estate and to determine the effect of varying inoculum density on biodegradation. High Performance Liquid Chromatography was used to monitor the biodegradation of the pesticides as well as both the formation and biodegradation of metabolites. HPLC data was then used to test three kinetic models of pesticide breakdown (First, Second and Third Order) to determine which could be used to best predict the biodegradation of the two pesticides. Using enrichment culture technique, one soil isolate was found capable of degrading Chlorpyrifos and another found capable of degrading Diuron. 16S rRNA gene sequence analysis revealed that the isolate capable of utilizing Chlorpyrifos as the sole carbon source was 95 % similar to *Kosakonia oryzae*, and the isolate's gene sequence was deposited in GenBank under the Accession Number MG517447. The isolate capable of utilizing Diuron as the sole carbon source was 92% similar to *Pseudomonas aeruginosa*, and the isolate's gene sequence was deposited in GenBank and assigned the Accession Number MG517448**.** For each isolate, High Performance Liquid Chromatography was used to monitor the pesticide and metabolite biodegradation at five different inoculum densities (1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml), with the initial pesticide concentration at 10 mg/L for Chlorpyrifos and 25 mg/L for Diuron. For Chlorpyrifos, maximum biodegradation of 100% was achieved with *Kosakonia oryzae* at the inoculum densities of 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12×10^8 CFU/ml within 8 days. For Diuron, maximum biodegradation of 83.59% was achieved at the inoculum density 6.0×10^8 CFU/ml within 18 days. Among the three kinetic models investigated, the First Order kinetic model best fit the data for biodegradation of both Chlorpyrifos and Diuron, but could not fully describe the process. This study documented for the first time the biodegradation of an organophosphate pesticide by *K.oryzae*. The study also investigated bacterial inoculum density as an independent variable in the kinetics of pesticide biodegradation. The two bacterial isolates obtained in this study are potential candidates for use in bioremediation of pesticide contaminated sites.

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ACRONYMS AND ABBREVIATIONS

EXTOXNET- Extension Toxicology Network

NSC- Nzoia Sugar Company

NRDB- Nzoia River Drainage Basin

HPLC- High Performance Liquid Chromatography

US EPA- United States Environmental Protection Agency

APVMA- Australian Pesticides and Veterinary Medicine Authority

CFU- Colony Forming Units

CHAPTER ONE

INTRODUCTION

1.0 Background

Pesticides are man-made xenobiotic chemicals and are widely used in most areas of crop production to minimize infestations by pests and thus protect crops from potential yield losses and reduction of product quality (Damalas and Eleftherohorinos, 2011). However, they have been proven to be harmful; once pesticides and their toxic residues enter the food chain, they affect not only humans but a diverse variety of organisms (Arau´jo *et al*., 2003). Knowledge about pesticide breakdown mechanisms and rates in soils is an important prerequisite to their safe and efficient use. While pesticides and their harmful by-products may be removed or dissipated from the environment in several ways, the role of microbes in the breakdown of pesticides and other pollutants in soil through bioremediation is significant (Pino and Penuela, 2011).

Bioremediation is the use of naturally occurring or deliberately introduced microbes to contaminated sites to break down or otherwise remove xenobiotic contaminants, in this case pesticides, from the environment. Bioremediation is potentially faster and cheaper than other methods of decontamination as it involves naturally occurring microorganisms. It is also safer since there is no use of harmful chemicals and the end products are benign molecules. As such, bioremediation as a technology is an effective avenue for removal of pesticides at contaminated sites worldwide, especially in developing countries (Boopathy, 2000). Factors affecting bioremediation of pesticides include environmental factors (temperature, pH, availability of sources of energy like carbon, moisture content), pesticide characteristics (molecular structure, physical and chemical attributes, concentration) and characteristics of the microbes involved (population diversity, enzyme activities and population size/concentration) (Boopathy, 2000). In the NRDB in Kenya, Chlorpyrifos and Diuron are among the pesticides used in sugarcane farming. Chlorpyrifos [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is one of the most widely used organophosphorous insecticides

worldwide (Solomon *et al*., 2014). The chemical structure of Chlorpyrifos is shown in Figure 1.1.

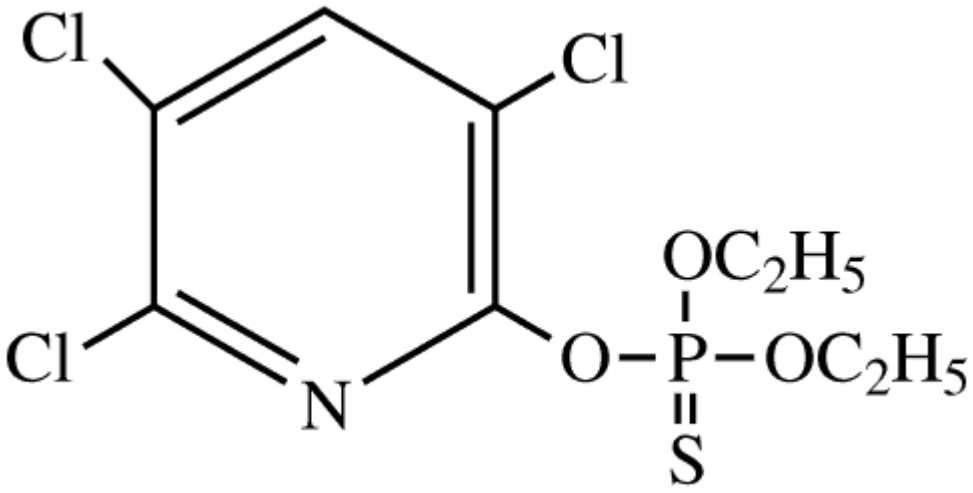

Figure 1.1: Chemical structure of Chlorpyrifos

(https://pubchem.ncbi.nlm.nih.gov/compound/Chlorpyrifos). **Visited 05/05/2018**

It is a broad-spectrum chlorinated organophosphate insecticide. It is used on a variety of food and feed crops to control a wide variety of insect pests found in soil and foliage (Solomon *et al*., 2014). It is used on fruit, grain, nuts, vegetables, livestock, ornamentals, golf courses, buildings, and for treating wood products. Diuron [*N*-(3, 4-dichlorophenyl)-*N*, *N*-dimethylurea] is a substituted phenylurea herbicide used to control a wide variety of annual and perennial broadleaf and grassy weeds, as well as some mosses. The chemical structure of Diuron is shown in Figure 1.2. It is heavily used worldwide e.g. in California, USA, over 1.4 million pounds were applied statewide in 2004 (US EPA, 2009).

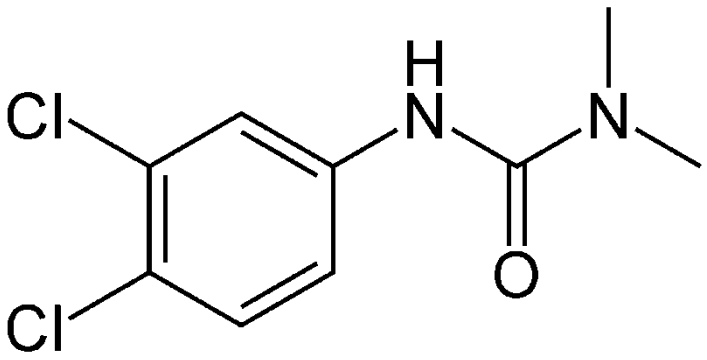

Figure 1.2: Chemical structure of Diuron

(https://pubchem.ncbi.nlm.nih.gov/compound/Diuron). Visited 05/05/2018

1.1 Pesticide use in the NRDB

Chlorpyrifos (CPF) is an organophosphate which is used on sugarcane in the NRDB (Mutua *et al*., 2015). Equally commonly used is Diuron which belongs to the phenylurea class of pesticides, (Ngigi *et al*., 2011). Both pesticides are used repeatedly, depending on season and emergence of pests, leading to a significant amount of both pesticides ending up in soil and groundwater, thus affecting non-target organisms, as reported by Omwoma *et al*., (2010). This has been the case for several years, as both pesticides have been in use for decades (Ngigi *et al*., 2011; Jemutai-kimosop *et al*., 2012; Mutua *et al*., 2015). CPF is used on termite mounds in the NRDB, under the trade name Pyrinex. Diuron is applied to the surface of soil, in between rows of sugarcane. It is used under the trade name Diurex.

1.1.1 Diuron Use in the NRDB

Diuron [*N*-(3, 4-dichlorophenyl)-*N*, *N*-dimethylurea] is a substituted phenylurea herbicide used to control a wide variety of annual and perennial broad-leaf and grassy weeds, as well as some mosses. It is indicated as a pre-emergence pesticide hence is applied to soil prior to emergence of weeds to control susceptible weed seedlings for an extended period of time. Diuron is applied at the rate of 5 kg a.i/ha (Jemutai-kimosop *et*

al., 2012) in the NRDB and has been used on sugarcane in the area for over two decades (Ngigi 2011). It adsorbs strongly to soil particles, meaning it persists in the environment for long periods of time (Sorensen *et. al* 2008). In addition to this, it has a moderate to low solubility in water, making it highly mobile. The NRDB experiences two rainy seasons in a year, with the mean annual rainfall varying from a maximum of 1100 to 2700 mm and a minimum of 600 to 1100 mm (Ngigi *et al*., 2011). This high amount of rainfall coupled with Diuron's high mobility increase the potential for widespread and far-reaching pollution by this pesticide in the region. Studies have reported detection of Diuron residues and metabolites in rivers in the NRDB and in Lake Victoria (Ngigi *et al*., 2011).

1.1.2 Chlorpyrifos Use in the NRDB

Chlorpyrifos, [O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum, widely used organophosphate pesticide. It is used in protection of agricultural crops such as sugarcane, coffee, tea, cocoa, rice, wheat, potatoes, vegetables, bananas, citrus fruits and cotton. It is also used in the protection of ornamental plants, domestic animals and built structures such as domestic houses and commercial establishments. In the NRDB, it is applied at the rate of 0.94 kg a.i/ ha (Mutua *et al*., 2015). CPF is used in the Nzoia Nucleus Estate primarily as a termiticide, where it is applied directly to termite mounds where they arise. It is usually mixed with organic liquids prior to use since it dissolves only moderately in water. Organophosphate pesticides cause an estimated 200,000 deaths annually (Chaou *et al*., 2013) and CPF is one of the most widely used organophosphate pesticides in Kenya (Manduu, 2015). CPF and its residues and metabolites have been reported in the NRDB (Mutua *et al*., 2015).

1.2 Statement of the Problem

Residues of Chlorpyrifos and Diuron have previously been detected in the NRDB (Mutua *et al.,* 2015; Ngigi *et al.,* 2011). Despite evidence of the detrimental effects of the two pesticides to both human beings and the ecosystem, efforts to phase out the two pesticides have so far failed or been only implemented in few countries (Trasande,2017; APVMA, 2005). In Kenya, there are currently no policies aimed at reducing the use of

these pesticides. There is also widespread sale and use of counterfeit pesticides in Kenya (PCPB, 2005). Over 650,000 people are at risk of pesticide poisoning in the NRDB, where the two pesticides are heavily used (NSC, 2015). This represents a major risk to both public and environmental health in the Nzoia sugar company Nucleus Estate and the NRDB at large.

1.3 Justification

In-situ bioremediation using bacteria resident in soil is a cost-effective and environmentally friendly method of removal of harmful pesticides from the environment. Isolation and characterization of pesticide degrading bacteria may thus be useful for bioremediation processes. Previous studies involving inoculum density as a factor affecting biodegradation concluded that high inoculum densities ($\geq 10^6$ to 10^8CFU/g of soil) of degrading bacteria are required for efficient biodegradation (Farhan *et al.*,2012). However, there is no information about specific optimum inoculum densities and time frames for biodegradation of Chlorpyrifos and Diuron and their metabolites. The bacterial densities required and time frames necessary vary according to the specific bacteria involved and their environment, and therefore this study generated information about the same, which can be used in bioremediation protocols using the bacteria in the same or similar areas and conditions.

1.4 Objectives of the Study

1.4.1 General Objective

To isolate, Characterize and Study Biodegradation Kinetics of Chlorpyrifos and Diuron-degrading Bacteria from Sugarcane Soils within the NRDB

1.4.2 Specific Objectives

1. To isolate Chlorpyrifos and Diuron- degrading bacteria from exposed agricultural soil in the NRDB
2. To determine the identities of Chlorpyrifos and Diuron- degrading bacteria from exposed agricultural soil in the NRDB using 16 S rRNA gene sequencing
3. To determine the kinetic effects of varying the bacterial inoculum density on the biodegradation of Chlorpyrifos and Diuron.

1.5 Hypotheses

i. There is no significant difference between Chlorpyrifos and Diuron degrading bacteria and other bacteria in exposed agricultural soil in the NRDB
ii. There is no significant difference between the identities of Chlorpyrifos and Diuron- degrading bacteria from exposed agricultural soil in the NRDB based on 16 S rRNA gene sequencing
iii. There is no significant difference in the kinetic effects of bacterial inoculum densities on the biodegradation of Chlorpyrifos and Diuron.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chlorpyrifos

Chlorpyrifos [O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum, widely used organophosphate pesticide. It is used in protection of agricultural crops such as sugarcane, coffee, tea, cocoa, rice, wheat, potatoes, vegetables, bananas, citrus fruits and cotton. It is also used in the protection of domestic animals and built structures such as domestic houses and commercial establishments (Singh and Walker, 2006). Chlorpyrifos (CPF) was introduced in 1965 by Dow Chemical Company and since then, it has been one of the most widely used organophosphate pesticides (Solomon *et al*., 2014). CPF exerts its insecticidal and pesticidal effects through inhibition of the enzyme cholinesterase, which acts on the neurotransmitter acetylcholine. It interferes with production of acetylcholine, thus affecting transmission of nerve impulses.

CPF is available in different formulations like spray, dust, emulsifiable concentrate, flowable, wettable powder, granular bait, pellet and microcapsule (US EPA, 2009). Organophosphorus pesticides have many sites in its structure which are vulnerable to hydrolysis, alkylation and dealkylation and oxidation because they are esters (Singh and Walker, 2006). Degradation of CPF in soil, animals, plants and mammals begins with cleavage of the phosphorus ester bond. Presence of the double bond at the central phosphorus atom is significant as it is the site of biotransformation of the molecule within tissues of some organisms, which affects its toxicity (Costa *et al*., 2003). Chlorpyrifos is a white to colorless solid with a boiling point of 320 ^{0}F at 760 mm Hg. It has a low solubility in water (0.39 mg/L at 19.5 ^{o} C). It is only slightly mobile in soils as it strongly adsorbs organic matter in soil (Jemutai-kimosop *et. al.,* 2012). However, volatilization increases its mobility. Its half-life in soil is 90-120 days and is therefore highly persistent.

2.2 Diuron

Diuron [*N*-(3, 4-dichlorophenyl)-*N*, *N*-dimethylurea] is a substituted phenylurea herbicide used to control a wide variety of annual and perennial broadleaf and grassy weeds, as well as some mosses and algae. It is used on crops such as sugarcane, pineapple, cotton, wheat, alfalfa, apples and in forestry, among others. It is also applied on rights-of-way and in drainage furrows and ditches when dry, as well as farm and industrial buildings (Cox 2003). Diuron is available in different formulations such as liquid suspension, granular, wettable powder, flowable, soluble concentrate, pellets and tablets. Phosphatidylcholine-clay (PC-clay) formulations are used as slow release formulations to help reduce environmental contamination (Undabeytia *et al.*, 2012). Diuron is a white crystalline solid with a Boiling Point of 356 to 374 ^{o} F at 760 mm Hg. Its solubility in water is 42 mg/L at 25 ^{o} C, and it has a very low solubility in hydrocarbon solvents. Due to this high solubility, it is highly mobile and easily leached by groundwater in soil. Its persistence in soil is 22- 49.5 days when applied at the recommended dosage.

2.3 Chlorpyrifos and Diuron Toxicity

The toxic effects of CPF stem from its inhibitory action on the enzyme acetylcholinesterase. This enzyme is involved in transmission of nerve impulses at the synapses of the central and peripheral nervous system, hence most of the pesticide's toxic effects are seen in the central nervous system, respiratory system and cardiovascular system. CPF binds irreversibly to the active site of acetylcholinesterase, thus inactivating the enzyme. CPF has also been found to inhibit Neuropathy Target Esterase enzyme. Inhibition of this enzyme affects the nervous system by causing axon degeneration and myelin sheath loss (Iyer *et al*., 2008). Acute exposure to CPF in humans causes symptoms such as ataxia, tremors, headaches, hypotension, drowsiness, respiratory depression and comas (Ballantyne and Marrs, 1992). It also causes peripheral neuropathies and polyneuritis in acute, high doses (Ballantyne and Marrs, 1992). It can also cause delayed neuropathy, which occurs one to three weeks after acute exposure to

CPF. This is partly because CPF is lipophilic and therefore binds to body fat, remaining in the body longer without being removed in urine. Symptoms of delayed neuropathy include cramps, weakness, numbing and tingling in the extremities, a high stepping gait, paralysis of the lower limbs and quadriplegia in some extreme cases. Chronic exposure to CPF causes symptoms such as weakness, malaise and loss of appetite (Occupational Health Services, 1991). Impaired memory, severe depression, insomnia and irritability have been observed in workers exposed to CPF repeatedly (Occupational Health Services, 1991). Hepatic biotransformation of CPF results in Chlorpyrifos oxon, an active metabolite of CPF that is more toxic than CPF itself (Iyer *et al*., 2008). Chlorpyrifos has been found to be toxic to wild animals and bees). In aquatic organisms like fish and aquatic invertebrates, CPF concentrations as low as 0.00454 kg of active ingredient per acre were reported to cause death. CPF is also toxic to birds, with the LD50 varying from one species to another. In some birds like mallards, CPF exposure can lead to reduced numbers of eggs laid (http://pmep.cce.cornell.edu/profiles/extoxnet/carbaryl-dicrotophos/Chlorpyrifos-ext.html)

Acute exposure to Diuron causes eye and throat irritation. Acute exposure on intact skin causes no significant symptoms apart from irritation when the dosage is low. Diuron is readily absorbed through the gut and lungs when inhaled or ingested orally. In studies using rats as models, the oral LD50 is 3.4g/kg (EXTOXNET, 2003). Diuron is categorized as a known/likely carcinogen due to increased incidences of bladder, kidney and breast cancer in rat models exposed to Diuron (US EPA 2003). Acute exposure to sub-lethal doses of Diuron causes formation of methaemoglobin, an abnormal form of the blood protein haemoglobin which carries oxygen. Methaemoglobin cannot carry oxygen. Acute exposure can lead to decrease in the number of red blood cells, increase in the number of abnormally shaped red blood cells, and increase the number of white blood cells. It can also cause hepatomegaly, which is enlargement of the liver due to the organ's role in detoxification. Diuron acute exposure may cause the spleen to become congested as it removes damaged red blood cells from circulation (Cox, 2003; EXTOXNET 2003). Juveniles are more susceptible to the harmful effects of acute exposure than adults (EXTOXNET 2003). Diuron was found to cause an increase in

Reactive Oxygen Species (ROS), which cause oxidative stress to cells (Huovenen *et al*., 2015). Diuron was also found to inhibit cell proliferation in human placental trophoblasts, thus potentially negatively affecting human fetuses (Huovenen *et al*., 2015). 3, 4 DCA, the main metabolite of Diuron breakdown, is more toxic than Diuron itself. It is readily absorbed through dermis, orally and by inhalation in rats and even more so in rabbits. Extrapolation of data from other aromatic amines indicates that humans are likely more sensitive to metheamoglobin formation than rats (EXTOXNET 2003).

Diuron and 3, 4 DCA are toxic to mammals like rabbits, but have been found non-toxic to bees. Diuron is moderately toxic to fish and birds but highly toxic to aquatic invertebrates (http://extoxnet.orst.edu/pips/Diuron.htm). Due to its high solubility and mobility, Diuron has negatively impacted several marine ecosystems. It has been reported to be partially responsible for the worst die-back of mangrove trees in history, and has been reported to be the most harmful pesticide in the Great Barrier Reef in Australia (APVMA 2005).

2.4 Environmental Effects of Chlorpyrifos and Diuron

The half-life of Chlorpyrifos greatly varies but is usually reported to be 90- 120 days (Liang *et. al.*,2011). This relatively high persistence is due to its low solubility in water and relatively strong adsorption to soil particles and other organic particles in soil. Its solubility in water is 0.0014 g/L (1.4 mg/L) at 25 °C and soil sorption coefficient (K_{oc}) 360 to 31,000 depending on soil and environmental conditions (John *et al*., 2015). Because CPF adsorbs strongly to soil particles, there is relatively low potential to leach to ground water (Liang *et al.*, 2011) or to be removed by surface runoff (Mary John *et al.*, 2015). This strong adsorption also shields CPF from dissipation by photolysis, thus leaving microbial degradation as the main avenue for dissipation from soil.

Diuron leaches more readily into deeper soil with less organic matter and has a high potential to contaminate groundwater. Diuron does not adsorb strongly to soil particles due to its low soil adsorption coefficient but adsorbs strongly to organic particles in soil (EXTOXNET, 2003). It has a relatively long hydrolysis half-life in soil, usually 90-180 days. However, the half-life has been reported to be as long as 300 days (US EPA

2009), meaning it is both highly persistent and mobile in the environment (Moncada *et al*., 2004). This means it cannot be controlled seasonally and when applied to soil, Diuron and its residues are commonly detected in the environment. In Australia, Diuron use has negatively impacted the Great Barrier Reef and has been found to be the main pesticide entering and impacting the Reef, mainly through runoff (http://www.wwf.org.au,2012). In France, Diuron has been detected in aquatic environments (Pesce *et al*., 2010) even after its use was banned in 2008. High mortalities of the Pacific oyster *Crassostreagigas* in coastal areas of France have been attributed to Diuron use as the pesticide has been found to affect the organism at the molecular and biochemical level (Luna-Acosta *et al*.,2012). In Japan, samples from the Kurose River were tested for the presence of a number of pesticides. Diuron was detected in samples from all sites, at a maximum concentration of 4620 ng/ L, higher than the other two pesticides (Kaonga *et al*., 2015). In Europe, the European Commission classifies Diuron as a substance of major concern in European waters as it has been detected in several watersheds (Stork *et al*., 2008, Schuler & Rand 2008). In California U.S.A, Diuron was detected in 418 water wells as reported in DPR's well inventory database WIDB as of 2004 (Moncada 2004). Diuron's main biodegradation product, 3,4dichloroaniline (DCA) has been reported to have a much higher toxicity (nearly 100 times) than Diuron (Giacomazzi and Cochet, 2004).

2.6 Biodegradation of Chlorpyrifos and Diuron

2.6.1 Biodegradation of Chlorpyrifos

Biodegradation of organophosphate pesticides by microbes is the single most important factor or process which determines their fate in the environment (Karpouzas *et. al*, 2005). Biodegradation of Chlorpyrifos mainly occurs through hydrolysis of the P-O alkyl or P-O aryl bond (Singh and Walker, 2006), as shown in Figure 2.1. Several bacteria and fungi have been found to have the ability to degrade CPF with varying degrees of efficiency (Yang *et al*., 2005; Obojska *et al*., 2002; Silambarasan and Abraham, 2013). While some bacteria degrade the pesticide into other metabolites,

others completely degrade the Chlorpyrifos into CO_2 and organic matter (see Figure 2.1 below for the degradation pathway) (Singh and Walker, 2006). Microbial degradation of Chlorpyrifos results in 3, 5, 6, trichloro-2-pyridinol (TCP) as a major metabolite (Robertson *et al*., 1998). CPF was previously reported to be resistant to enhanced degradation, unlike other organophosphates, due to the antimicrobial activity of the resultant 3, 5, and 6- trichloro- 2- pyridinol (TCP). TCP represses the proliferation of microbes, thus limiting CPF degradation (Racke *et al*., 1990). However, microbes have been reported which have the ability to mineralize the antimicrobial metabolite TCP. These bacteria have molecular and biochemical attributes that allow them to tolerate TCP or to mineralize TCP at a rate higher than the rate of its formation. Examples of bacteria capable of degrading both CPF and TCP are *Alcaligens faecalis* strain DSP3 (Yang *et al*., 2005), *Providencia stuartii* strain MS09 (Rani *et al*., 2008) and *Cupriavidus sp*. DT-1 (Lu *et al*., 2013).

2.6.1.1 Molecular Basis of Chlorpyrifos Biodegradation

Genes involved in hydrolysis of Chlorpyrifos are opd genes and mpd genes. These genes code for organophosphate degrading enzymes. The opd gene codes for the enzyme organophosphorus hydrolase and have been isolated from several organisms. *P.diminuta* MG (Serder *et al*., 1982) and Flavobacterium *sp* ATCC 27551(Mulbry *et al*., 1986), the opd genes were found located in plasmids. The opd gene in *Agrobactrium radiobacter* was found to be located on the chromosome (Horne *et al*., 2002). The mpd gene was isolated by Cui *et al*., (2001) from *Plesiomonas sp*. M6. In *Pseudomonas sp*. WBC-3, the mpd gene is located on a plasmid (Liu *et al*., 2005). The mpd gene has been found to be located in the chromosome in strain YC-1, which belongs to the genus *Stenotrophomonas*, as no plasmid was detected in this strain of bacteria (Yang *et al*., 2006). The gene was successfully cloned into *E. coli* DH5α cells, using a recombinant plasmid pMDQ as a vector, thus showing that the degradative trait is transferrable (Yang *et al*., 2006).

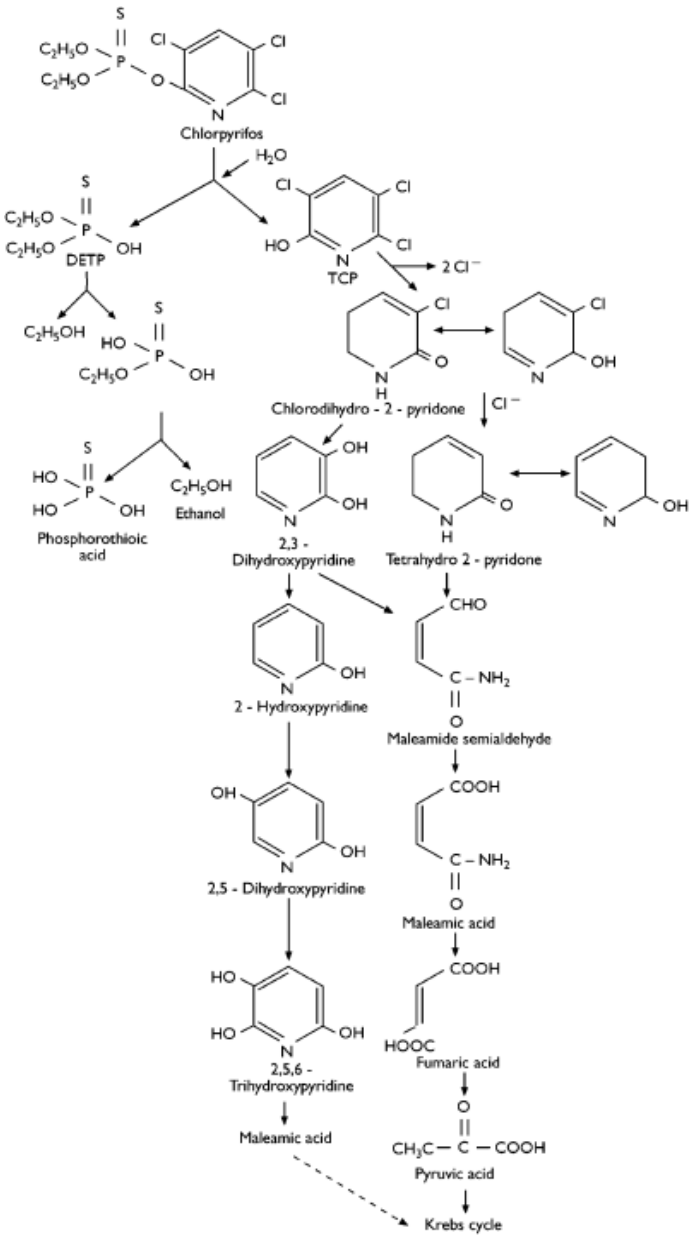

Figure 2.1: Pathways of CPF Biodegradation in different Microorganisms

2.6.2 Biodegradation of Diuron

Under aerobic conditions Diuron biodegradation usually occurs through N-demethylation followed by ring cleavage. The products are 3, 4 dichloroaniline (DCA); N-(3,4-dichlorophenyl)-N-methylurea (DCPMU); 3,4-dichlorophenylurea (DCPU), with DCA being the main metabolite (Figure 2.2) (Ellegaard-Jensen *et. al*, 2013). Figure 2.2

shows biodegradation of Diuron (aerobic), including different microbes and their biodegradation actions (Ellegaard-Jensen et. al, 2013)

2.6.2.1 Molecular Basis of Diuron Biodegradation

Two gene/enzyme systems involved in Diuron hydrolysis are PUH A (*puhA*/PuhA) and PUH B (*puhB*/PuhB). These systems code for the enzyme phenylurea hydrolase, which hydrolyses the carbon-Nitrogen bond in phenylurea herbicides. The enzyme belongs to a metal-dependent amidohydrolase superfamily (It has a Zn^{2+} active site) and contains an Asn-X-His metal-binding motif (Khurana *et al*.,2009). The molecular basis of Diuron biodegradation was studied in *Arthrobacter globiformis* strain D47 (Turnbull *et al*., 2001). *Arthrobacter globiformis* strain D47 is a soil microbe that rapidly hydrolyses Diuron and other phenylurea herbicides. In this microbe, biodegradation was found to occur through hydrolysis of the urea carbonyl group (Turnbull *et al*., 2001). The gene involved here is PUH A. Plasmid curing, DNA profiling and purification showed that hydrolytic genes are localized on the 47-kb plasmid (pHRIM620). Since the Diuron-degrading trait is plasmid based, it can be transferred to other cells using vectors. PUH B was characterized from *Mycobacterium brisbanense* strain JK1 (Khurana *et al*., 2009). Repeated Diuron treatments of soil result in increased puhB copy numbers in soil bacterial communities (Pesce *et al*., 2013), thus the more a soil is exposed to Diuron treatments, the higher the genetic potential of its microbes to degrade the pesticide.

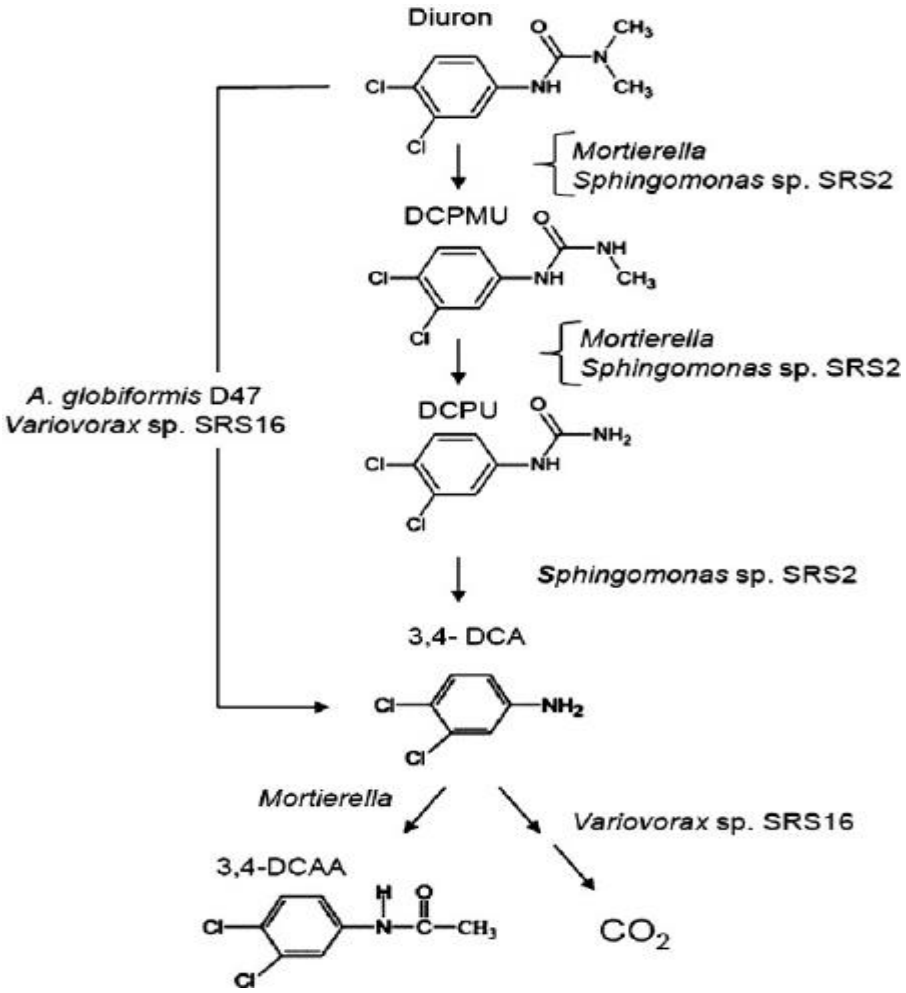

Figure 2.2: Degradation Pathways of Diuron

2.7 Effect of Bacterial Inoculum Density on Chlorpyrifos and Diuron Biodegradation

Several bacteria have been shown to have the ability to degrade Chlorpyrifos. For bioremediation using microbial inoculation to be carried out effectively; the microbes in question must be present in a sufficient quantity (Farhan *et al*., 2012). Strain DSP3 when introduced to soil at a bacterial density of 10^8 cells/g of soil treated with 100 mg/kg of Chlorpyrifos, degraded nearly 100% of Chlorpyrifos in the soil in 20 days (Yang *et al*., 2005). An addition of strain DSP3 to soil resulted in a higher degradation rate than non-inoculated soils. The inoculation of strain YC-1 at a bacterial density of 10^6 cells/g of soil treated with 100 mg/kg Chlorpyrifos resulted in a higher degradation rate than in non-inoculated soils (Yang *et al*., 2006). With *Enterobacter sp* B-14 in

liquid medium, degradation was slow initially at cell densities of $<10^4$ cells mL^{-1}, but the lag phase was followed by rapid degradation. At high inoculum density ($>10^4$ cells mL^{-1}), Chlorpyrifos was degraded completely within 24 h (Singh and Walker, 2006). Farhan *et al*., (2012) reported that 94% of Chlorpyrifos was degraded in 18 days when the inoculum size of *Pseudomonas sp* used was 10^8 CFU/ml. Inoculum density of 10^3 CFU/ml degraded 47% of the pesticide, suggesting an almost linear relationship between the density of degrading bacteria and the extent of biodegradation of the pesticide. There appear to be minimum bacterial densities below which biodegradation and hence bioremediation cannot occur effectively. With *Enterobacter sp*. there was no observable degradation of Chlorpyrifos below a cell density of 103 cells/ g (Singh and Walker, 2006). Singh and Walker, (2006) found that generally, for effective in-situ bioremediation of Chlorpyrifos, a bacterial density of 10^6-10^8 cells/g of soil is necessary.

Several bacteria have been reported to have the ability to degrade Diuron (Turnbull *et al*., 2001, Widehem *et al*., 2002; Sorensen et al 2008). *Actinobacter baumannii* was reported to achieve mineralization of 27% Diuron with bacterial density of 2×10^6 CFU/g of soil (Dellamatrice *et al*., 2001). *A.globiformis* D47 degraded over 90% of Diuron in 10 days at a bacterial density of 1.2×10^7 CFU/ g of soil.

2.8 Kinetic Considerations in Biodegradation

Biodegradation kinetics generates degradation curves which allow us to better understand the effect of different factors on the rate of biodegradation. They also allow us to predict the course of biodegradation under different conditions (Jonsson and Haller, 2014). In earlier studies, biodegradation processes of CPF (Bondarenko *et al*., 2004; Singh and Walker, 2006) and Diuron (Sharma *et al*., 2010) were found to follow the First-Order decay model, in which the rate of degradation of the pesticide is proportional to the concentration.

The first-order degradation kinetics may be expressed as follows

$$dC/dt = -k1\ C$$

Where C- Concentration of a degraded compound at the time t

k1 - First-order rate constant

The first-order rate constant is often replaced by a half-life (H) and the degradation rate is then expressed as follows (Dąbrowska *et al*., 2004)

$$dC/dt = - (0.6933/H) C$$

$$\text{Where } H = 0.693/k1$$

In the study by Singh and Walker, (2006), a positive linear correlation (R^2=0.98) between cell densities of Chlorpyrifos-degrading bacteria and biodegradation of Chlorpyrifos supported the first order kinetic theory. This occurred under optimized and varying incubation conditions (pH 7, 30 ^{o}C, with shaking). For Diuron, the relationship between cell densities of degrading bacteria and biodegradation has been reported to follow first order kinetics up to a certain value (Sharma *et al*., 2010). These studies, however, used the initial concentration of the pesticide as the varying factor. At present, there exist few studies involving bacterial population size/ density as a varying factor in generating kinetic data for CPF and Diuron biodegradation. This study aimed to generate this information. Kinetic constants are function of the type and concentration of the pollutant to be treated and also depend on the microorganisms effecting the degradation. No pesticide biodegradation studies involving varying inoculum densities have been done using soil bacteria isolated from the soils of the NRDB. This study aimed to generate that information. In regards to bacterial pesticide biodegradation, Single First Order kinetics assume that the number, or in this case density, of degrading bacteria and their associated enzymes is large relative to the number of pesticide molecules. As a result, the levels of the pesticide decrease at a steady rate. The differential form of the Single First Order equation is

$$\text{Rate} = -\left(d[A]/dt\right) = k[A] \ldots. \quad (1)$$

Rearranged, this gives:

$$d[A] / [A] = -kdt \ldots. \quad (2)$$

After integrating both sides of equation (2) and applying the calculus rule that $\int (1/x) = \ln(x)$, we get:

$$\ln[A] - \ln[A]o = -kt \ldots. \quad (3)$$

Rearranging to solve for [A] gives:

$\ln[A]=\ln[A]o-kt$.... (4)

Rearranging equation (4) into y=mx +b form:

$\ln[A]=-kt+\ln[A]o$ …. (5)

The equation is a straight line with slope m:

$mx=-kt$

and y-intercept b:

$b=\ln[A]o$

Where [A] is the concentration of the pesticide at time t and [A]o is the concentration at time 0, and k is the first-order rate constant. The slope of the graph gives -k. As logarithms have no units, $-kt$ lacks units and it follows that the unit of k in a First Order reaction is time^{-1}

The Second Order rate law is as follows

$d[A] / dt=-k[A]$…. (1)

When this equation is integrated, it gives

$1 / [A]=1/ [A]o+kt$…. (2)

Equation (2) iny=mx +b form:

$1 / [A]= kt + 1/ [A]o$.... (3)

y= 1 / [A]

m= k

b= 1 / [A]o

Where [A] is the concentration of the pesticide at time *t* and [*A*]*o* is the concentration at time 0, and *k* is the Second-Order rate constant. The slope of the graph gives *k.*

Under the Third Order model, the rate of the reaction is proportional to three concentration terms. The rate of the reaction is given by the equation

Rate of the reaction=k $[A]^3$

The half-life time is inversely proportional to the square of the initial concentration of the reactant.

Focusing on biodegradation of fixed concentrations of CPF and Diuron, with varying inoculum densities of respective bacteria, the three kinetic models for pesticide biodegradation were studied, namely First Order, Second Order and Third order.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Research Design and Methodology

Bacterial isolates capable of biodegrading CPF and Diuron were isolated from exposed agricultural soil from the study area. Bacterial isolates were obtained from soil via enrichment culture technique. Pesticide-degrading isolates were identified using conventional Microbiology, Biochemical tests and 16 S rRNA analysis. The effect of varying inoculum densities on the biodegradation of the respective pesticides was performed and studied using High Performance Liquid Chromatography.

3.1 Study Area

This study was carried out using soil from the Nzoia Sugar Company Nucleus Estate within the NRDB as shown in Figure 3.1. The Nzoia Sugar Company is one of the largest sugar companies in Kenya. In its Nucleus Estate, the company has over 3600 ha under sugarcane. The Nucleus Estate farms lie between longitudes 34º34'00"-34º51'30"E and latitudes 0º23'00"-0º37'30"N. The soil type in the sugarcane-growing area is friable volcanic clay with mean annual rainfall being 1100-2700mm. The wet season runs from February-March and the dry season is November-December. Rivers Nzoia and Kuywa are the two main rivers that run through the Nucleus Estate. Apart from CPF and Diuron, the pesticides carbofuran and hexazinone are also widely used in sugarcane farming in the area.

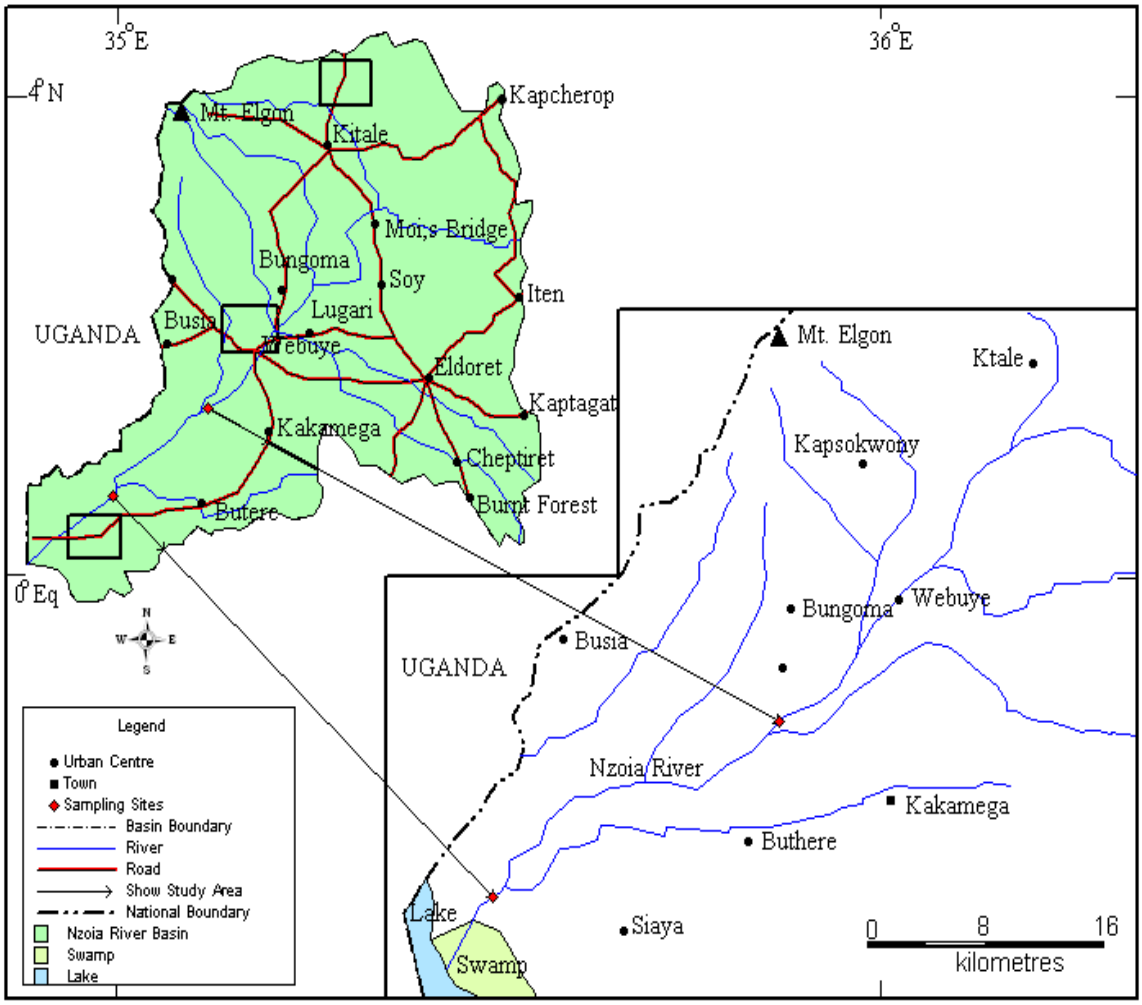

Figure 3.1: Map of study sites within the NRDB

3.2 Sampling

The soil sampling sites were located in the Nzoia Sugar company Nucleus Estate, lying between longitudes 34º34'00"-34º51'30" E and latitudes 0º23'00"-0º37'30" N. Plots and sites used in sampling were identified using random stratified sampling technique. The sites selected were those with a long history of exposure to the pesticides (> 1 year). For Chlorpyrifos, soil was collected from termite mounds in and around sugarcane fields. Soil was collected from the surface of the termite mounds as well as from deep within the mounds, since the pesticide was applied both on the surface of the mounds

and sprayed into holes in the mounds. For Diuron, soil was collected from the surface of fields, from a depth of 0-15cm, since the pesticide was applied only on the top surface of soil to kill weeds. All the soil samples were placed in sterile Whirl Pak bags then placed in a cooler at 4^{o} C for transport to the laboratory. 60 soil samples were collected for each pesticide.

3.3 Reagents and Chemicals

Chlorpyrifos and Diuron analytical standards (≥99.5% purity) were obtained from Sigma-Aldrich Corporation. Analytical standards of the metabolites TCP, 3, 4-DCA, DCPU and DCPMU were also obtained from Sigma-Aldrich Corporation. Tryptic Soy Broth Medium (TSB medium), Nutrient Agar, MacConkey Agar and 5% Agar-agar were obtained from Sigma-Aldrich Corporation. All reagents were prepared according to the manufacturer's instructions. Acetonitrile and water used in HPLC analysis were of HPLC grade. Chlorpyrifos Mineral Salt Medium (MSM) was constituted as follows (in grams per liter of distilled water): $(NH_4)_2NO_3$, 1.0; Ca $(NO_3).2H_2O$, 0.04; $MgSO_4.7H_2O$, 0.1; KCl, 0.2; $FeSO_4 \cdot 7H_2O$, 0.001, $K_2HPO_4 \cdot 12H_2O$, 1.5; and KH_2PO_4, 4.8 and 1ml trace metal solution. The pH was brought to 7.0 using a scientific Benchtop pH meter (Rani *et al*., 2008). The MSM was then autoclaved at 121 ^{o}C for 15 minutes. Addition of Chlorpyrifos to the media was done by aseptically dissolving Chlorpyrifos in a minimal volume of HPLC-grade methanol and adding it to the MSM solution at a concentration of 100mg/L. Diuron Mineral Salt Medium was constituted as follows (in grams per litre of distilled water): $NaNO_3$, 6.0; $KH_2\ PO_4$, 1.5; KCl, 0.5; $MgSO_4$.7H2O, 0.5; $FeSO_4$, 0.001; $ZnSO_4$, 0.001 and 1ml trace metal solution. The pH was brought to 6.8 using a scientific Benchtop pH meter (Dellamatrice *et al*., 2004).

3.4 Isolation of Pesticide-degrading Bacteria

This section details the procedures followed in the isolation of Chlorpyrifos and Diuron degrading bacteria from soil.

3.4.1 Isolation of CPF-degrading Bacteria

Isolation of Chlorpyrifos-degrading bacteria from soil samples was carried out by enrichment culture technique using a modification of the method described by Ifediegwu *et al*., (2015). Isolates were screened for their ability to utilize the pesticides by growing them on pesticide mineral salt agar following the method of Ifediegwu *et al*., (2015). Soil samples were crushed and air-dried, then passed through a 2-mm sieve. Ten 50ml bijou bottles were sterilized by autoclaving at 121 ^{o} C for 15 minutes. Five of these bottles were filled with the MSM and five filled with TSB medium. 10 g soil samples were weighed and added to the solution in each bijou bottle. All the bottles were placed in a Wisecube rotational shaker at 120 revolutions per minute (rpm) for 48 hours at 37 ^{o} C. At 12-hour intervals, one bottle each of CPF-enriched MSM and TSB medium were removed from the shaker. From each bottle, a loop-full of solution was inoculated on nutrient agar and MacConkey agar using a sterile wire loop. The nutrient agar and MacConkey agar plates were incubated at 37 ^{o} C for 24 hours. CPF mineral salt agar were prepared by first preparing a 5% Agar-agar solution and sterilizing it by autoclaving at 121 ^{o} C for 15 minutes then aseptically adding CPF as the only carbon source (dissolved in minimal volume of HPLC-grade methanol) at a concentration of 10mg/L. All the bacteria which grew on the nutrient agar and MacConkey agar plates were transferred to CPF mineral salt agar using a sterile wire loop. CPF mineral salt agar plates were incubated for 7 days at 37 ^{o} C until single colonies were observed. The plates were observed after every 24 hours for the appearance of single colonies. Soil from Kakamega Forest with no history of CPF exposure was used as a control. Isolates were stored at -80 ^{o} C in tryptic soy broth with 15% glycerol for subsequent experiments.

3.4.2 Isolation of Diuron-degrading Bacteria

Isolation of Diuron-degrading bacteria from soil samples was carried out by enrichment culture technique using a modification of the method used by Ifediegwu *et al*., (2015) in isolating Chlorpyrifos degrading bacteria from soil. Soil samples were crushed and air-dried, then passed through a 2-mm sieve. Ten 50ml bijou bottles were sterilized by autoclaving at 121 ^{o} C for 15 minutes. Five of these bottles were filled with the enriched

MSM and five filled with TSB medium. 10 g soil samples were weighed and added to the solution in each bijou bottle. All the bottles were placed in a Wisecube rotational shaker at 120 revolutions per minute (rpm) for 48 hours at 37 ^{o}C. At 12-hour intervals, one bottle each of Diuron-enriched MSM and TSB medium were removed from the shaker. From each bottle, a loopful of solution was inoculated on nutrient agar and MacConkey agar. The nutrient agar and MacConkey agar plates were incubated at 37^{0} C for 24 hours. Diuron mineral salt agar were prepared by first preparing a 5% Agar-agar solution and sterilizing it by autoclaving at 121 ^{o} C for 15 minutes then aseptically adding Diuron as the only carbon source (dissolved in minimal volume of HPLC-grade methanol) at a concentration of 25mg/L. Single colonies which grew on the nutrient agar and MacConkey agar plates were transferred to Diuron mineral salt agar using a sterile wire loop. Diuron mineral salt agar plates were incubated for 14 days at 37 ^{o} C until single colonies were observed. The plates were observed after every 24 hours for the appearance of single colonies. Soil from Kakamega Forest with no history of Diuron exposure was used as a control. Isolates were stored at -80 ^{o} C in tryptic soy broth with 15% glycerol for subsequent experiments.

3.5 Identification of Pesticide-degrading Isolates

Pesticide degrading isolates were identified using conventional microbiology, biochemical tests and 16 S rRNA analysis according to previously described methods as detailed in the subsequent sub-sections (Rani *et al*., 2008).

3.5.1 Biochemical Tests

Bacterial isolates were subjected to gram staining and subsequent biochemical tests. The tests performed were Motility, Catalase, Voges-Proskauer, Methyl Red, Indole production, Citrate, Oxidase, Nitrate, H_2S, Urease, Starch hydrolysis, Glucose fermentation, Sucrose and Lactose fermentation.

3.5.2 Bacterial DNA Extraction and PCR Amplification of 16SrRNA

Genomic DNA from the bacterial isolates was extracted using Qiagen microbial DNA extraction kit following the manufacturer's protocol. The protocol utilized the selective binding properties of the silica membrane in the kit to isolate pure DNA. After lysis in an optimized buffer and adjustment of DNA binding conditions, the sample was loaded directly onto a QIAamp spin column. DNA was bound to the silica membrane, and contaminants were completely removed in 2 wash steps. Pure DNA was eluted in small volumes of a low-salt buffer The DNA obtained was contaminant-free and ready for use in downstream applications. The universal primers 27F (5'AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACGGCTACCTTGTTACGACTT 3') were used in PCR amplification of the extracted DNA. The amplification step of PCR was carried out in reaction solutions containing the following: 0.5 µL of 27F primer (200ng/µL), 0.5 µL of 1492R primer (200ng/µl), 2.5 µL10X PCR reaction buffer, (100mMTris-HCl, 500 mM KCl, pH 8.3), 1.5 µL 25 mM$MgCl_2$ solution, 4.0 µL 1.25 mM, dNTPs, 0.1 µL AmpliTaq Gold DNA polymerase enzyme and 2 µL of DNA as the template. The reaction volumes were made up to 25 µL using sterile ultrapure water. The profile of thermal cycling consisted of an initial denaturation step at 94 ^{o}C for 3 min, followed by 30 cycles of denaturation (for 1 min at 94 ^{o}C), annealing for 1 min at 57 ^{o}C, and extension for 2 min at 72 ^{o}C, followed by a final extension for 8 min at 72 ^{o}C. As a safeguard against false positives which may arise from reagent contamination, negative controls were included. Amplicons were separated on 1.5% agarose gels in 1XTBE buffer at 10V cm^{-1} for 30 minutes. After this, the amplicons were stained with Ethidium bromide and observed using a BioRad UV transilluminator. The gel was photographed and the bands in it were located using a UV lamp. The bands were cut out and placed in a 2mL eppendorf tube. The PCR fragments were then extracted from the gel using Qiagen Gel extraction kit following the manufacturer's protocol.

3.5.3 Sequencing Reactions of the 16SrRNA

Sequencing reactions were performed at Bioneer, South Korea using the BigDye Terminator v3.1 sequencing Kit (Applied Biosystems, USA) with the primers 27F and 1492R. 12 µl of (4 µl ss DNA, 2 µg, 4 µl 0.8 µM primer, 2 µl 10x MOPS buffer and 2 µl 10x Mn^{2+} isocitrate buffer) was added in 1.5ml microcentrifuge tube, then incubated at 65-70 ^{o} C for 5 minutes to denature DNA and allow primers anneal. The reaction was allowed to cool at room temperature for 15 minutes, and then briefly centrifuged to reclaim condensation. To each reaction, 22 µl (7 µl ABI terminator mix (401489), 2 µl diluted Sequenase [TM] (3.25 U/µl), and 1 µl 2 mM a-S dNTPs) was added and incubated for 10 minutes at 37 ^{o} C before 20 µl 9.5 M ammonium acetate and 100 µl 95% ethanol was added and vortexed. It was then centrifuged again for 15 minutes, and carefully the supernatant decanted. DNA was then precipitated in ice-water bath for 10 minutes, centrifuged for 5 minutes at 12000 rpm in a microcentrifuge at 40C and supernatant carefully decanted and rinsed in 300 µl of 70-80% ethanol. DNA was then dried for 5-10 minutes in the Speedy-Vac. Thermal-cycling Conditions included 60^{0}C for 30 minutes and holding at 4 ^{o} C. Sequenced products were analyzed in an automatic sequencer, ABI3730XL DNA Analyzer (Applied Biosystems).

3.6 Effect of Varying Bacterial Isolate Densities on Pesticide Degradation

For CPF, single colonies of bacterial isolates successfully growing on CPF mineral salt agar were transferred to nutrient agar using a sterile wire loop. After 24 hours, single colonies were transferred to liquid shake cultures at 25 ^{o} C in 50 ml of Mineral salt Medium supplemented with 10 mg/L of Chlorpyrifos, pH 7.2 in 125 ml Erlenmeyer flasks. Bacterial growth was monitored by measuring the turbidity at A_{600}until an OD_{600} of 0.669 was obtained, corresponding to MacFarland Standard no. 4 (12×10^{8}CFU/ml) (Clinical and Laboratory Standards Institute, 2012). This suspension was then compared to a MacFarland Standard no. 4 prepared in the laboratory for visual confirmation. At the mid- log phase (20 hours), viability was measured and 1 ml of the MSM culture was harvested by centrifugation at 10 000 r per minute. The pellet was then washed once

with sterile 0.85% saline solution and resuspended in 5 ml of sterile 0.85% saline solution. Dilutions were prepared in 0.85% saline solution in test tubes with bacterial densities corresponding to 9.0×10^8CFU/ml ,6.0×10^8CFU/ml, 3×10^8CFU/ml and 1.5×10^8CFU/ml, corresponding to MacFarland Standard nos.3, 2, 1, and 0.5 respectively, with each test tube having 1ml of bacterial suspension. Each dilution was compared to the corresponding MacFarland Standard prepared in the laboratory for visual confirmation.

For Diuron, single colonies of bacterial isolates successfully growing on Diuron mineral salt agar were transferred to nutrient agar using a sterile wire loop. After 24 hours, single colonies were transferred to liquid shake cultures at 25 ^{o} C in 50 ml of Mineral salt Medium supplemented with 25 mg/L of Diuron, pH 7.2 in 125 ml Erlenmeyer flasks. Bacterial growth was monitored by measuring the turbidity at A_{600}until an OD_{600} of 0.669 was obtained, corresponding to MacFarland Standard no. 4 (12×10^8CFU/ml). This suspension was then compared to a MacFarland Standard no. 4 prepared in the laboratory for visual confirmation. At the mid- log phase (20 hours), viability was measured and 1 ml of the MSM culture was harvested by centrifugation at 10 000 rpm. The pellet was then washed once with sterile 0.85% saline solution and resuspended in 5 ml of sterile 0.85% saline solution. Dilutions were prepared in 0.85% saline solution in test tubes with bacterial densities corresponding to 9.0×10^8CFU/ml ,6.0×10^8CFU/ml, 3×10^8CFU/ml and 1.5×10^8CFU/ml, corresponding to MacFarland Standard nos.3, 2, 1, and 0.5 respectively, with each test tube having1ml of bacterial suspension. Each dilution was compared to the corresponding MacFarland Standard prepared in the laboratory for visual confirmation as shown in table 3.1.

Table 3.1: MacFarland Standard Preparation

MacFarland Standard	1% $BaCl_2$ (ml)	1%H_2SO_4 (ml)	Approximate bacterial Suspension/mL
0.5	0.05	9.95	1.5×10^8CFU/ml
1	0.10	9.90	3.0×10^8CFU/ml
2	0.20	9.80	6.0×10^8CFU/ml
3	0.3	9.7	9.0×10^8CFU/ml
4	0.4	9.6	12×10^8CFU/ml

Legend: The table describes the preparation of MacFarland standards. The reaction between $BaCl_2$ and Sulphuric acid yields Barium sulphate, which makes the solution turbid. Different amounts of the reactants produce different quantities of this product, leading to varying degrees of turbidity.

Dilution Plate Count Method was then used to confirm the bacterial densities. The Dilution Plate Method is used to determine the number of viable bacteria in a fixed amount of liquid. It utilizes the principle of serial dilution. Starting from a plate with an easily countable number of colonies, and taking into account the dilution factor, the number of bacteria in the original culture can be determined. The procedure was repeated for bacteria which had successfully tolerated Diuron salt agar. To each test tube with 1ml bacterial suspension, 9ml of enriched CPF MSM or enriched Diuron MSM was added bringing the total volume to 10 ml. Control test tubes had 9ml CPF or Diuron MSM with no bacterial suspension. The test tubes were incubated for time periods corresponding to the length of time which bacteria took to grow on their respective pesticide mineral salt agar. For both pesticides, biodegradation was investigated at five varying inoculums densities, namely 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12×10^8 CFU/ml. For CPF, the process of biodegradation was monitored over a period of 8 days. Aliquots of 2ml samples were removed for HPLC analysis at time intervals of 0, 2,4,6,7 and 8 days. Readings were taken in triplicate and the average values and Standard deviations calculated and

graphs constructed. For Diuron, the process of biodegradation was monitored over a period of 18 days. Aliquots of 2ml samples were removed for HPLC analysis at time intervals of 0, 4, 8, 12, 16 and 18 days. Readings were taken in triplicate and the average values and Standard deviations calculated and graphs constructed.

3.7 Pesticide & Metabolite Residue Extraction and Analyses of Samples by High Performance Liquid Chromatography (HPLC)

An aliquot of 2ml of enrichment culture was taken in separate flasks and an equal volume of acetonitrile added to each flask. The flasks were shaken vigorously using a reciprocating shaker for 30 minutes at 150 rpm. After this, samples were micro filtered through 0.2 μm nylon membrane filters to remove bacteria. Micro filtered samples were injected into the HPLC instrument in aliquots of 10 μL. Chlorpyrifos and its metabolites were detected at 240 nm using a mobile phase of HPLC grade acetonitrile and 1mM phosphoric acid at a ratio of 75:25 (v/v) respectively. Diuron and its metabolites were detected at 250 nm using a mobile phase of HPLC grade acetonitrile and double distilled water at a ratio of 70:30 (v/v) respectively with an isocratic flow at 1 ml per minute.

3.7.1. First- Order Rate Model

To test if it the biodegradation reactions fitted the First-Order model, plots were made for the natural logarithm of the pesticide concentration versus time to see whether the graphs were linear. A negative slope on a linear graph meant the biodegradation reaction was a First-Order reaction.

3.7.2 Second Order Rate Model

To test if it the biodegradation reactions fitted the Second-Order model, plots were made for the reciprocal of the pesticide concentration versus time to see whether the graphs were linear. A positive slope with a positive intercept on a linear graph meant the biodegradation reaction fitted the Second-Order model.

3.7.3 Third Order Rate Model

To test if it the biodegradation reactions fitted the Third-Order model, plots were made for the reciprocal of the square of pesticide concentration versus time to see whether the graphs were linear. Linear graphs with high R^2 values indicated a fit to Third order kinetic

CHAPTER FOUR

RESULTS

4.1 Isolation of Pesticide-degrading Bacteria

60 soil samples were analyzed for each pesticide. 39 bacteria were isolated and from these, two bacterial isolates- one capable of growing on Chlorpyrifos mineral salt agar and one capable of growing on Diuron mineral salt agar- were obtained from sample soils. Control soil from Kakamega forest, which had never been exposed to either pesticide, had no bacteria capable of growing on either pesticide mineral salt agar. The isolate labeled 7 successfully utilized Chlorpyrifos as the sole source of carbon, while isolate 21 successfully utilized Diuron as the sole source of carbon. Physical examination of the isolates gave results as shown in table 4.2.

4.2 Biochemical Tests

Bacterial isolates were observed growing on nutrient agar plates at the 18-hour growth stage for physical and morphological attributes. The Biochemical tests performed are shown in table 4.1. The results showed that isolate 7 was a rod-shaped, gram-negative bacterium. It was found to be motile, with the ability to ferment glucose to 2, 3 butanediol and to utilize citrate as a source of carbon. It also had the ability to utilize lactose and sucrose. These results pointed to isolate 7 being a member of the enterobacteriaceae family of gram-negative bacteria. For isolate 21, the bacterium was found to be gram- negative and motile, with the ability to utilize citrate as a carbon source and ability to convert tryptophan to indole. It also had the ability to reduce nitrate to nitrite. These results, coupled with the distinctive greenish color observed on plates, pointed to the isolate likely being *Pseudomonas aeruginosa*. Plates showing the results of biochemical tests are in Appendix D.

Table 4.1: Biochemical Characteristics of Chlorpyrifos and Diuron-degrading Bacteria

Test/ Observation	Isolate 7	Isolate 21
Physical Characteristics	Color-Whitish-cream; Margin-Entire; Texture-smooth; Elevation- Flat	Color-Greenish; Margin-Entire; Texture-soft; Elevation- Flat
Morphological Characteristics	Circular, Convex	Circular
Gram's Reaction	- (Rods)	- (Rods)
Growth on MacConkey agar	-	+
Voges-Proskauer	+	-
Methyl Red	-	-
Motility	+	+
Indole	-	+
Citrate	+	+
Catalase	+	+
Oxidase	-	+
Nitrate	-	+
H_2S	-	-
Urease	-	-
Lactose	+	-
Glucose fermentation	+	-
Sucrose	+	-

Legend: The table shows the results of visual examination and biochemical testing of the pesticide -degrading isolates. The tests were done as a first step in identification of the Isolates.

4.3 Genomic DNA Isolation and PCR Analyses

Genomic DNA was successfully extracted and PCR analyses done for both isolates.

4.4 Sequence Identity of Isolates

Quality control (Cleaning) was performed on sequence reads and consensus sequences were generated in Bioedit 7. The sequences were deposited in GenBank and BLAST search (BLASTN) was then performed in the GenBank database. The results are shown in table 4.2

Table 4.2: NCBI Accession numbers and BLAST Top Hits

Sample Label	NCBI Accession number	BLAST Top Hits
Isolate 7	MG517447	*Kosakonia oryzae*
Isolate 21	MG517448	*Pseudomonas aeruginosa*

Legend: The table shows the Accession numbers of isolate sequences and BLASTN top hits after sequence comparison between the bacterial isolates and nucleotide sequences in the NCBI database. From NCBI BLASTN, Isolate 7 was most similar to a strain of *Kosakonia oryzae*, while Isolate 21 was most similar to a strain of *Pseudomonas aeruginosa.*

4.5 16 S rRNA Partial Gene Sequences of Bacterial Isolates

The consensus partial gene sequences of the isolates were viewed in Bioedit 7. Plate 4.2 shows the sequence of Isolate 7 (1458 bp) and Plate 4.3 shows the sequence of Isolate 21 (1417 bp)

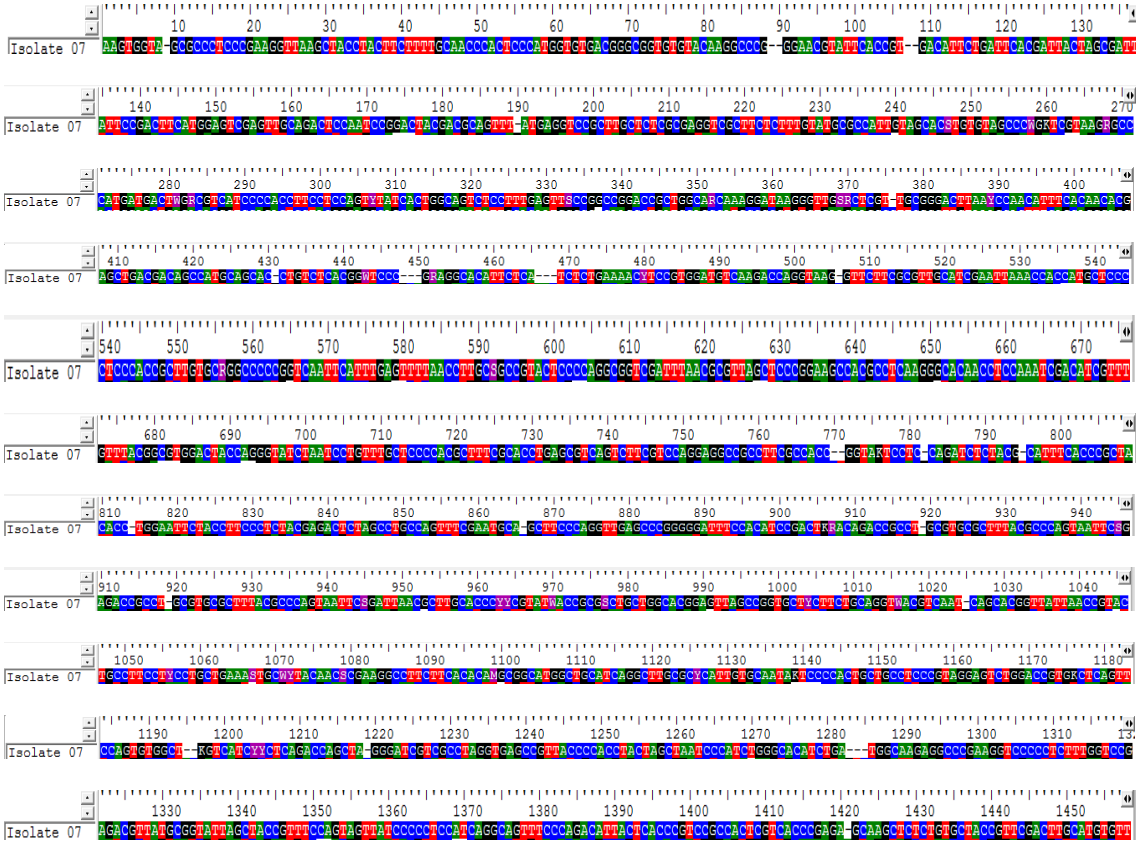

Plate 4.1: 16 S rRNA partial Gene Sequence for Isolate 7

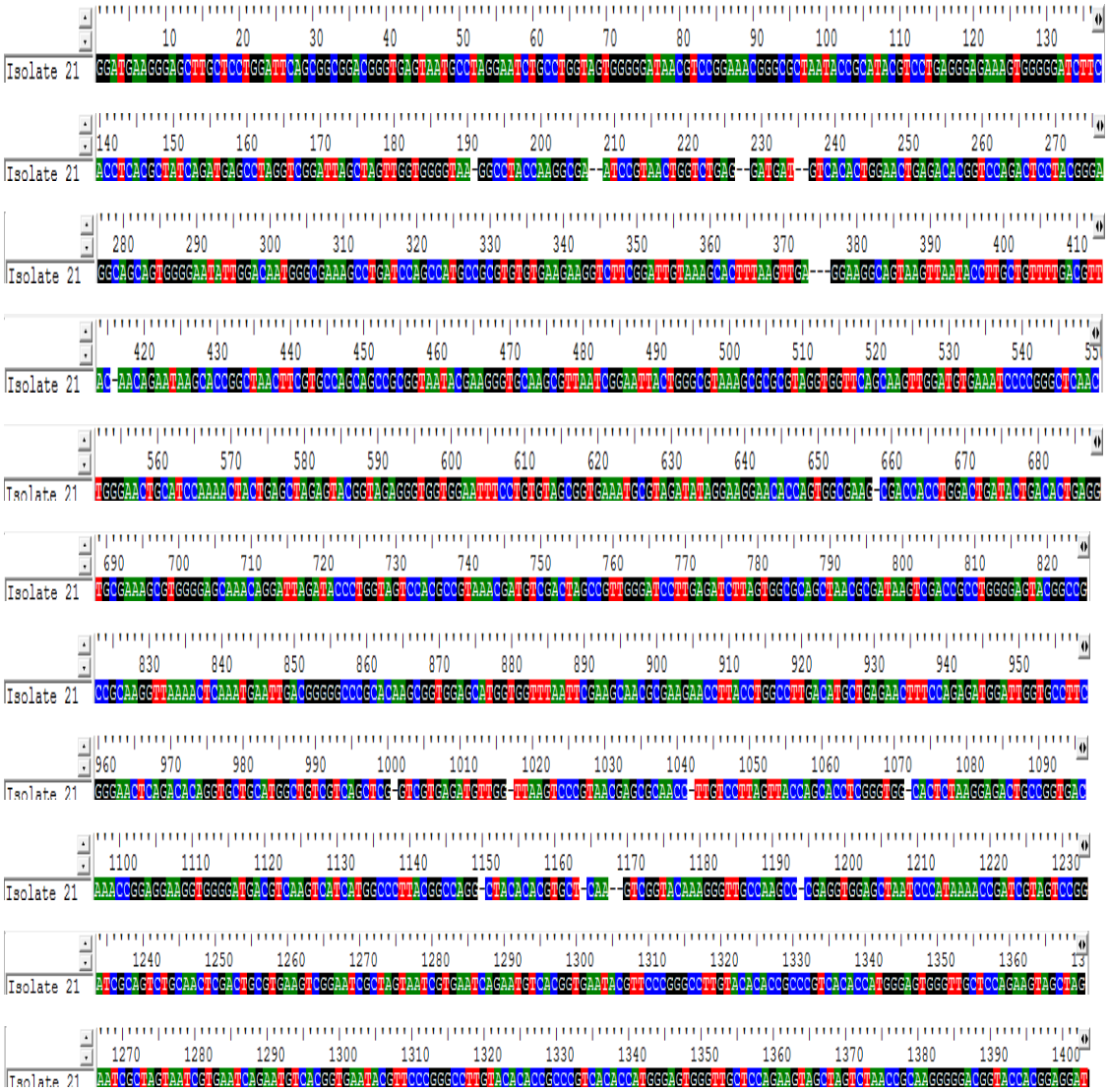

Plate 4.2: 16 S r RNA partial Gene Sequence for Isolate 21

4.6 Degradation of Chlorpyrifos and Diuron Under Laboratory Conditions

The biodegradation of the two pesticides Chlorpyrifos and Diuron was determined at a constant pesticide concentration at varied bacterial inoculum densities. High Performance Liquid Chromatography was used to analyze the levels of pesticides and their metabolites at different time intervals. For each of the two pesticides, the initial pesticide concentration was the same for each sample, with the bacterial inoculum density being varied.

4.6.1 Degradation of Chlorpyrifos and Metabolite Formation

The results showing degradation of Chlorpyrifos and its metabolites are shown in figure 4.4. Degradation of Chlorpyrifos was observed achieving complete removal within 8 days. 3, 5, 6 Trichloropyridinol (TCP), the principal biodegradation product of Chlorpyrifos, was detected and its formation and biodegradation in solution was monitored. It was observed that the bacterial isolate was able to degrade both Chlorpyrifos and TCP. TCP was produced from day 0 to day 2 and degraded from day 0 to day 8. TCP was completely degraded at the three highest inoculums densities of 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12×10^8 CFU/ml after 8 days. Maximum TCP biodegradation was observed at the highest inoculum density of 12×10^8 CFU/ml after 8 days. The results are shown in figure 4.4.

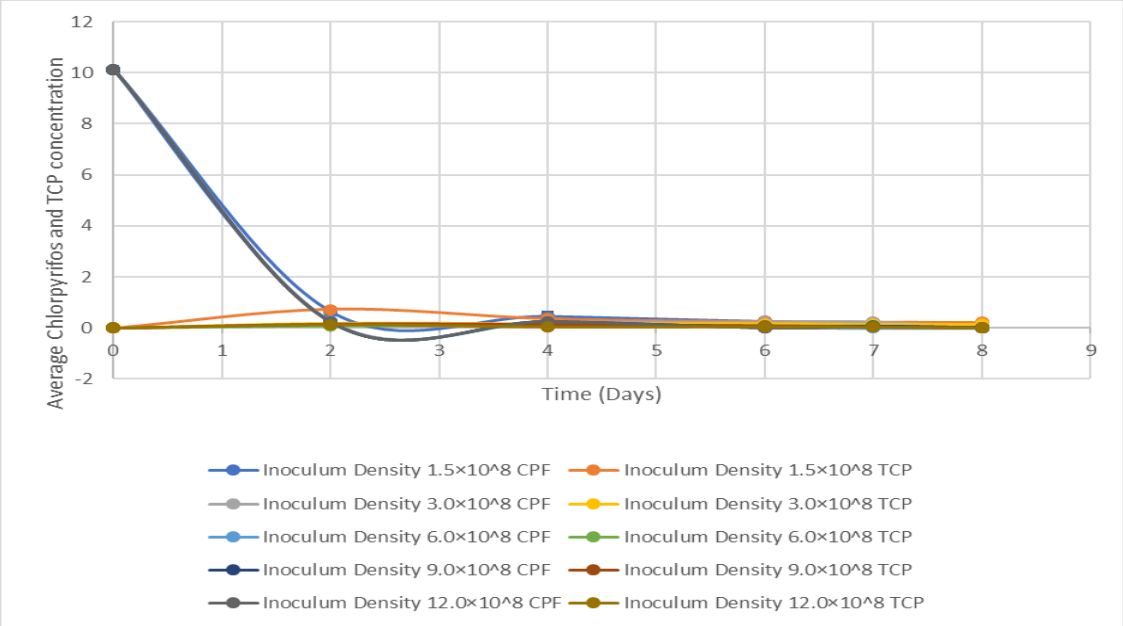

Figure 4.4: Biodegradation of Chlorpyrifos and TCP at different Inoculum Densities

As shown in figure 4.4, there was rapid biodegradation of Chlorpyrifos between day 0 and day 2 at all bacteria inoculum densities. However, concentration differences in concentrations were observed among all inoculum densities. Complete biodegradation of Chlorpyrifos (100%) was observed after 7 days for the three highest inoculum

densities of 6.0×10^8 CFU/ml,9.0×10^8 CFU/ml and 12×10^8 CFU/ml after 8 days. At the three highest inoculum densities (6.0×10^8 CFU/ml,9.0×10^8 CFU/ml and 12×10^8 CFU/ml) TCP produced was completely mineralized within the 8-day time frame. At all the inoculum densities, there was initial transient accumulation of TCP, with its levels reaching their maximum after 2 days.

4.6.2 Degradation of Diuron and Metabolite Formation

As shown in figure 4.5, Diuron levels were observed to decrease with time. Maximum Diuron biodegradation of 83.59% was observed at the inoculum density of 6.0×10^8 CFU/ml after 18 days. Diuron metabolites DCPMU and 3,4 DCA were not detectable after 18 days.

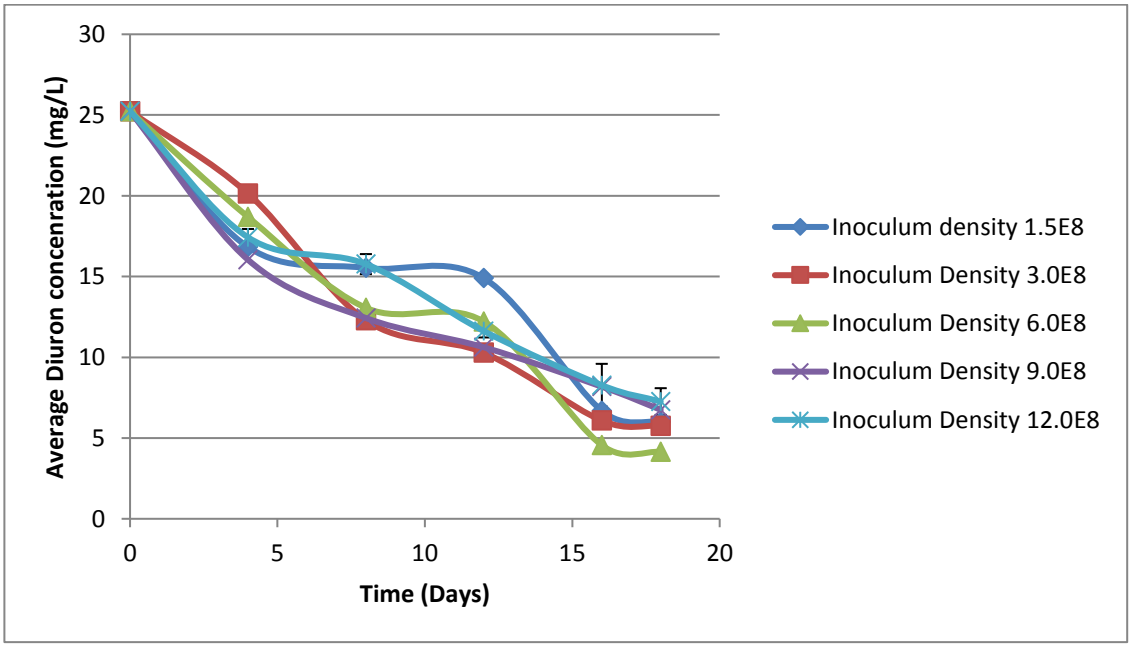

Figure 4.5: Biodegradation of Diuron at different Inoculum Densities

4.7 Kinetics of Effects of Varying Inoculum Densities on Pesticide Biodegradation

HPLC biodegradation data was tested against different pesticide degradation kinetic models to ascertain which best fit the data. The models tested were First Order, Second Order and Third Order.

4.8 Chlorpyrifos Kinetics

Chlorpyrifos kinetic data used in the plotting of graphs and subsequent calculations is found in the Appendix A. The R^2 values for each inoculum density are shown on the graphs and tabulated in table 4.3

4.8.1 Chlorpyrifos First Order Kinetics

Figure 4.6 shows the First order kinetics for Chlorpyrifos degradation. The R^2 values for each inoculum density under the First order model for Chlorpyrifos were calculated graphically and are displayed. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

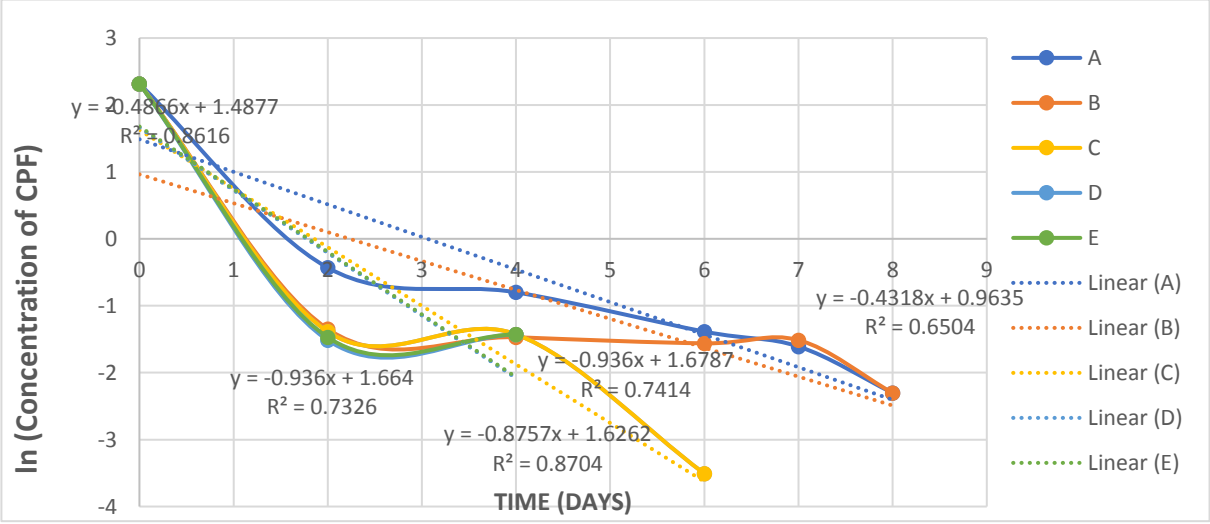

Figure 4.6: Chlorpyrifos First Order Degradation for the five Inoculum Densities

4.8.2 Chlorpyrifos Second Order Kinetics

Figure 4.7 details the Second Order Kinetics for Chlorpyrifos. The R^2 values for each inoculum density under the Second Order model for Chlorpyrifos were calculated graphically and are displayed in figure 4.7. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

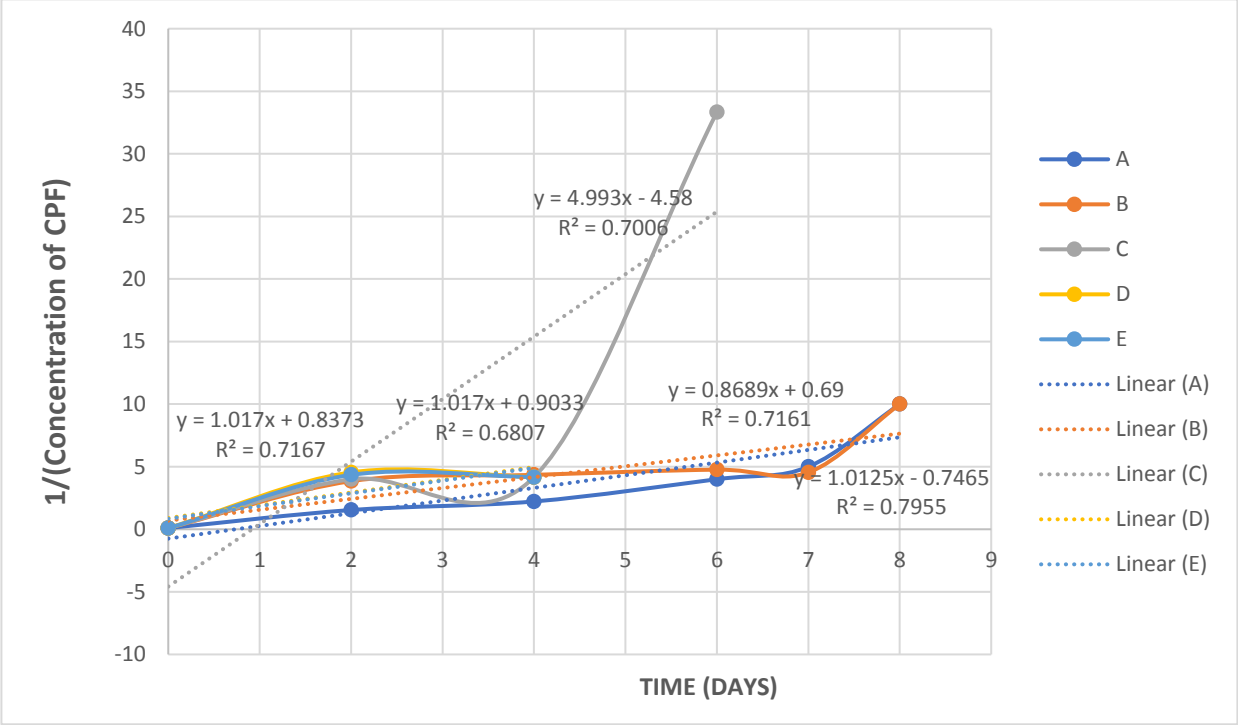

Figure 4.7: Chlorpyrifos Second Order Degradation for the five Inoculum Densities

4.8.3 Chlorpyrifos Third Order Kinetics

The R^2 values for each inoculum density under the Third Order model for Chlorpyrifos were calculated graphically and are displayed on each graph as shown in 4.8. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

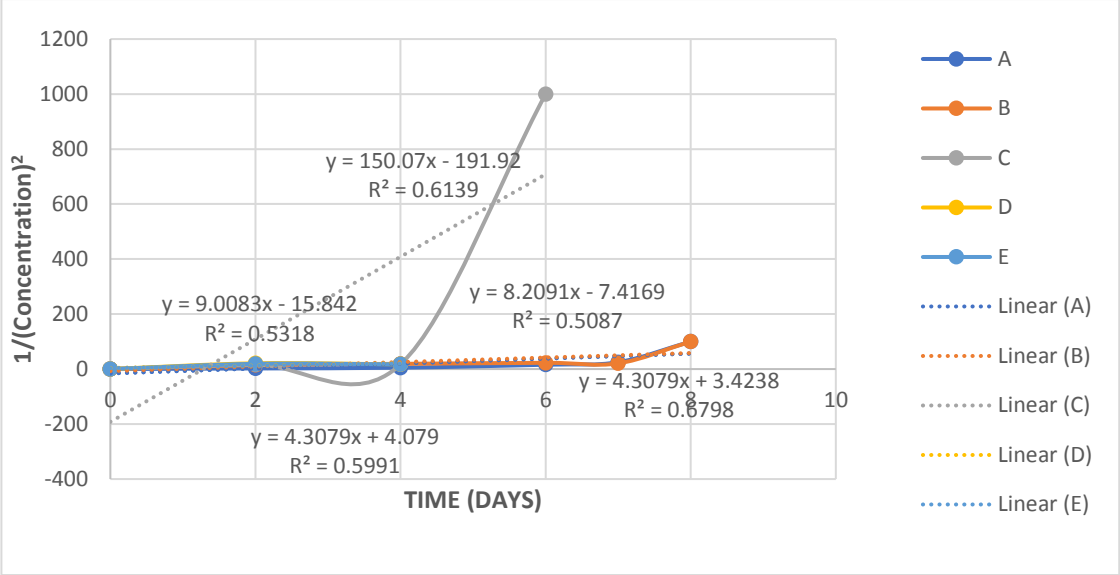

Figure 4.8: Chlorpyrifos Third Order Degradation for the five Inoculum Densities

Table 4.3: R^2 values for Chlorpyrifos Kinetic Biodegradation Models

Inoculum Density	R^2 Value		
	First Order	**Second Order**	**Third Order**
1.5×10^8 CFU/ml	0.861	0.795	0.531
3.0×10^8 CFU/ml	0.650	0.716	0.508
6.0×10^8 CFU/ml	0.870	0.700	0.613
9.0×10^8 CFU/ml	0.732	0.680	0.599
12.0×10^8 CFU/ml	0.741	0.716	0.679

Legend: The table shows R^2 values as derived from kinetic graphs for Chlorpyrifos degradation at different inoculum densities. Higher R^2 values indicate a better fit to the corresponding kinetic order of reaction.

4.9 Diuron Kinetics

Diuron kinetic data used in the plotting of graphs and subsequent calculations is found in the Appendix B. The R^2 values for each inoculum density are shown on the graphs and tabulated in table 4.4

4.9.1 Diuron First Order Kinetics

The R^2 values for each inoculum density under the First order model for Diuron were calculated graphically and are displayed on each graph as shown in figure 4.9. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

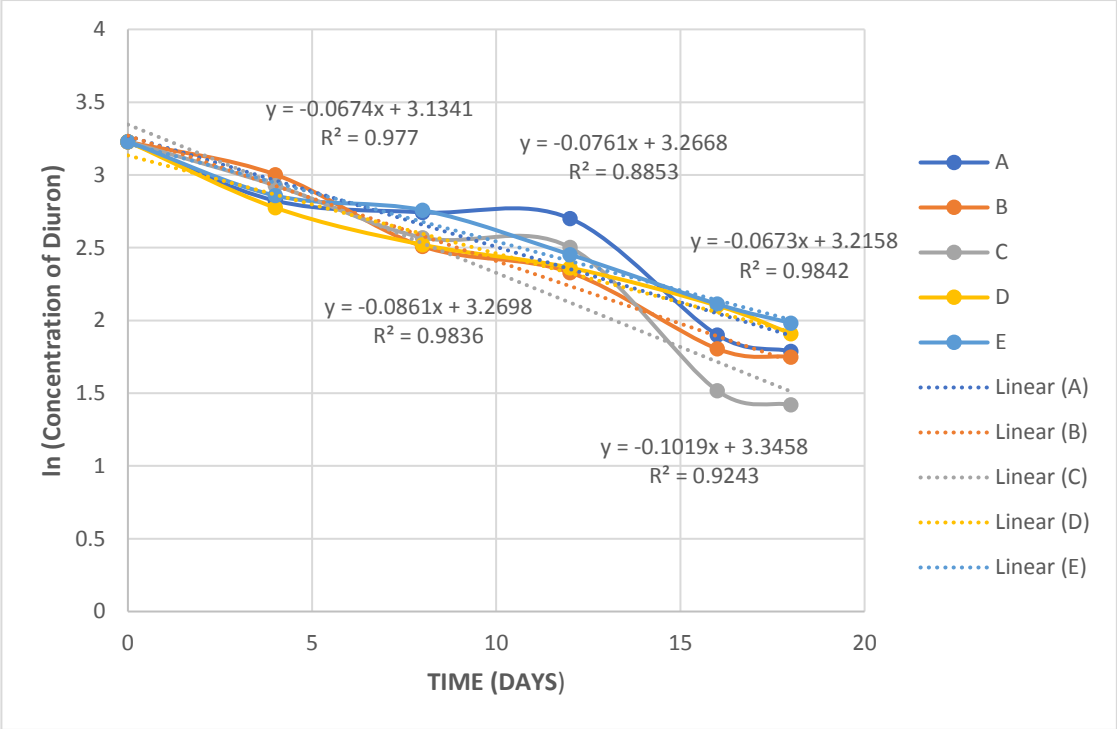

Figure 4.9: Diuron First Order Degradation for the five Inoculum Densities

4.9.2 Diuron Second Order Kinetics

The R^2 values for each inoculum density under the Second order model for Diuron were calculated graphically and are displayed on each graph as shown in figure 4.10. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

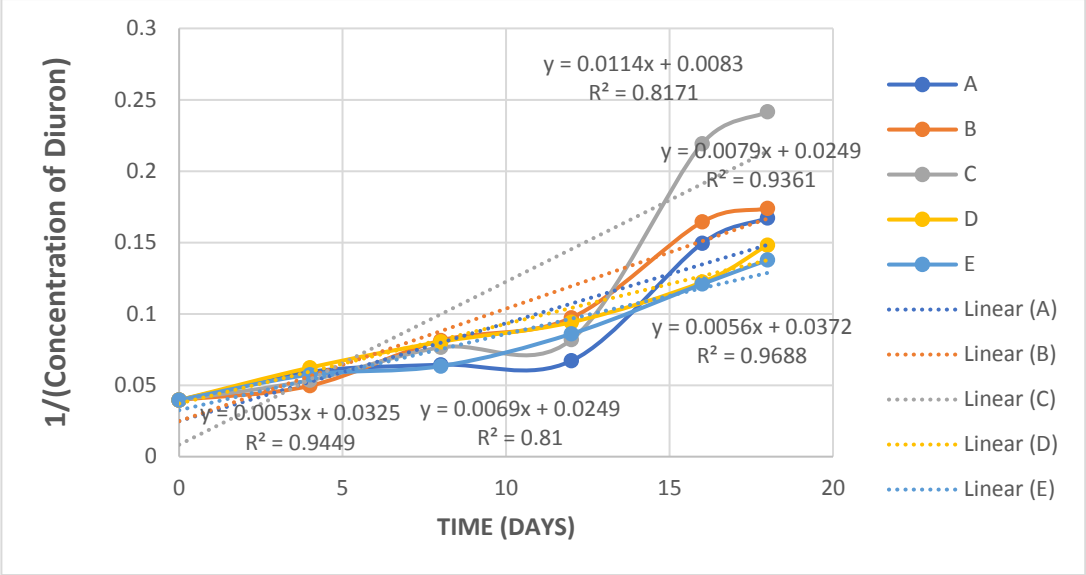

Figure 4.10: Diuron Second Order Degradation for the five Inoculum Densities

4.9.3 Diuron Third Order Kinetics

The R^2 values for each inoculum density under the Third Order model for Diuron were calculated graphically and are displayed on each graph as shown in figure 4.11. A to E represent the five inoculum densities of 1.5×10^8 CFU/ml, 3.0×10^8 CFU/ml, 6.0×10^8 CFU/ml, 9.0×10^8 CFU/ml and 12.0×10^8 CFU/ml.

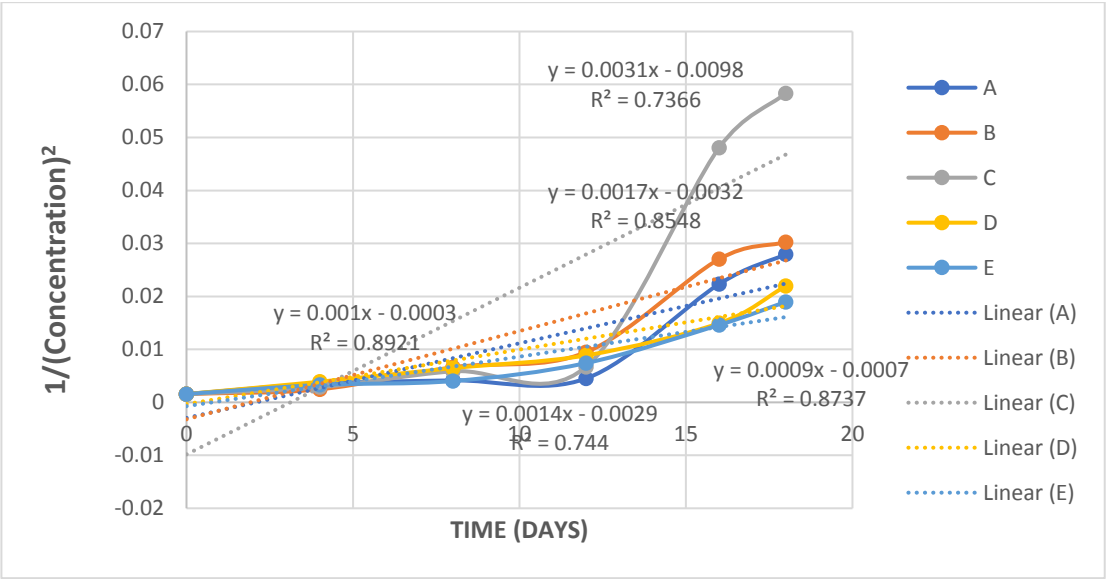

Figure 4.11: Diuron Third Order Degradation for the five Inoculum Densities

Table 4.4: R^2 values for Diuron Kinetic Biodegradation Models

Inoculum Density	**R^2 Value**		
	First Order	**Second Order**	**Third Order**
1.5×10^8 CFU/ml	0.885	0.81	0.744
3.0×10^8 CFU/ml	0.983	0.936	0.854
6.0×10^8 CFU/ml	0.924	0.817	0.736
9.0×10^8 CFU/ml	0.977	0.968	0.892
12.0×10^8 CFU/ml	0.984	0.944	0.873

Legend: The table shows R^2 values as derived from kinetic graphs for Diuron degradation at different inoculum densities. Higher R^2 values indicate a better fit to the corresponding kinetic order of reaction.

CHAPTER FIVE

DISCUSSION

Soil samples from the NRDB yielded 39 morphologically distinct bacterial isolates when the non- selective nutrient agar and MacConkey agar were used as growth media. These isolates were then subjected to the enrichment culture technique to obtain pesticide degrading isolates. The enrichment culture technique is a relatively simple approach to isolating pesticide degrading bacteria from soil. The basis of the technique is providing a growth medium with substrate(s) that only the microbe or organism of interest can utilize and other microbes or organisms cannot. This therefore makes the medium selective for the microbe or organism of interest. One isolate was found capable of utilizing Chlorpyrifos (Isolate 7) and one was found capable of utilizing Diuron (Isolate 21). For the Chlorpyrifos-degrading bacterium, observable growth on mineral salt agar occurred after a period of 7 days, while for Diuron- degrading bacterium, observable growth occurred after 14 days, showing that the CPF-degrading bacterium was likely more efficient at degrading CPF than the Diuron-degrading bacterium was at degrading Diuron. Perennial use of Chlorpyrifos and Diuron resulted in repeated exposure of soil bacteria to the pesticides over long periods of time. This exposure resulted in the bacteria developing the capability to utilize the pesticides as sources of carbon, evidenced by their ability to visibly grow on media with the respective pesticides as the only sources of carbon, a trait not seen with soil bacteria from unexposed soil. This capability likely arose through genetic adaptation, since biodegradation processes of both pesticides have been shown to be catalyzed by enzymes encoded by genes as described in studies elsewhere (Yang *et al.*,2006; Turnbull *et al.*,2001).

During characterization of the two pesticide-degrading isolates, the isolates were subjected to gram staining and subsequent biochemical tests, followed by 16S rRNA gene analysis. The tests performed were Motility, Catalase, Voges-Proskauer, Methyl Red, Indole production, Citrate, Oxidase, Nitrate, H_2S, Urease, Starch hydrolysis, Glucose fermentation, Sucrose and Lactose fermentation. The Motility test was used to determine if the bacterial isolate being tested possessed flagella (Prescott *et al.*, 2004). Both isolates were found to be motile. The Catalase test was used to determine whether

the isolate involved produced the enzyme catalase, which breaks down hydrogen peroxide to oxygen and water (Black, 2004). Both isolates were found to possess catalase enzyme. The Voges-Proskauer test and Methyl Red test were done in conjunction to determine the glucose fermentation pathway used by the bacterial isolate involved. The Voges-Proskauer test was used to detect the presence of acetoin produced during fermentation of glucose to 2, 3 butanediol, with a positive test indicating that the bacterial isolate involved fermented glucose to 2, 3 butanediol. The Methyl Red test was used to determine the presence of organic acids, with a positive test indicating that the bacterial isolate involved fermented glucose to organic acids (Prescott *et al.,* 2004). Isolate 7 was found to be positive for the Voges-Proskauer test, thus fermenting glucose to 2, 3 butanediol. Both isolates gave a negative result for the Methyl Red test.

The Indole Test was used to determine the presence of the enzyme tryptophanase in the isolate under test (Prescott *et al.,* 2004). This enzyme converts the amino acid tryptophan to indole, with a positive test indicating that the bacterial isolate involved was able to break down tryptophan. Isolate 21 was found to possess tryptophanase, while isolate 7 did not. The Citrate test was used to determine whether the bacterial isolate involved could utilize citrate as a carbon source, breaking it down through a series of steps into pyruvic acid and CO_2 (Prescott *et al.,* 2004). Both isolates were capable of utilizing citrate. The Oxidase test was used to determine the presence of cytochrome oxidase enzyme in the isolate, an enzyme in the terminal step of the electron transport chain (Black, 2004). Isolate 7 was oxidase-negative and isolate 21 oxidase-positive. The Nitrate test was used to determine whether the bacterial isolate involved could reduce nitrate to nitrite using nitrate reductase enzyme. Isolate 7 was nitrate reductase-negative and isolate 21 nitrate reductase- positive. The H_2S test was used to determine whether the bacterial isolate involved could break down Sulphur in the amino acid cysteine to H_2S (Prescott *et al.,* 2004). The Urease test was used to assess the bacterial isolate's ability to break down urea using urease enzyme (Prescott *et al.,* 2004). Both isolates were unable to break down sulphur and urea. The biochemical tests results pointed to isolate 7 likely being in the family Enterobacteriaceae. Biochemical tests for isolate 21 pointed to it being Pseudomonas spp. Further identification was necessary for both isolates to further characterize them up to at least species level.

Due to its ubiquitous nature as a highly conserved gene sequence in bacteria, the 16S rRNA gene sequence was analyzed to give further insight into the identity of the environmental isolates. Analysis of the isolate 7's 16S rRNA gene sequence using Nucleotide BLAST showed that it was closely related to *Kosakonia oryzae* strain Ola 51^T. The highest 16S rRNA gene sequence similarity (following comparison of the 16S rRNA gene sequence) of isolate 07 was found with *Kosakonia oryzae* strain Ola 51^T (95 % similarity). *Kosakonia oryzae* is a gram negative, rod-shaped non-spore forming nitrogen fixing bacterial species isolated from surface sterilized roots belonging to the wild rice species freely growing in China (Li *et al.,* 2017). It was found to be positive for acetoin production (Voges-Proskauer test) while negative for indole production; positive for β- galactosidase and arginine dihydrolase while negative for lysine decarboxylase; positive for oxidation of arabinose, cellobiose, citrate, fructose, galactose, gluconate, glucose, glycerol, lactose, malate, maltose, mannitol, mannose, sorbitol, sucrose and trehalose in previous studies (Peng *et al.,* 2009; Brady *et al.,*2013). Though this bacterium is aerobic, it was known to reduce N_2 to NH_3 at low pO_2 concentration (Li *et al.,* 2017). Most of these characteristics were found true with isolate 7 confirming that isolate 7 was indeed *Kosakonia oryzae*. There is currently no documented knowledge existing that links *Kosakonia oryzae* to the biodegradation of Chlorpyrifos or other organophosphate pesticides.

A widely distributed organophosphate-degrading gene (opd) was identified in geographically and biologically different species in studies by Richins (Richins *et al.,* 1997) and Wang (Wang *et al.,* 2002) but *Kosakonia oryzae* was not one of them. To the best of our knowledge, there is no single study that has found the existence of opd gene in this species. Previous studies in the NRDB have shown CPF-degrading bacteria are present in the soil (Mutua et al., 2015). This might suggest that *Kosakonia oryzae* found in this study had obtained Chlorpyrifos degrading genes from other opd positive bacteria found in soil, since the gene has been found in other soil bacteria like those discussed by Singh and Walker (Singh and Walker, 2006) which utilize organophosphates as major carbon sources. Isolate 21 was able to grow in the media containing Diuron pesticide as a sole carbon source. This indicated that it has the ability to degrade this environmental pollutant. Widehem *et al*. (Widehem *et al.,*2000) previously isolated microorganisms

able to degrade Diuron though this degradation resulted in the formation of the harmful metabolite 3,4-dichloroaniline which was further observed to be degraded by the fungi studied by Widehem et al. (Widehem *et al.*,2000) and *Arthrobacter sp*. bacteria studied by Tixier et al. (Tixier *et al.*,2002). In an attempt to identify and characterize isolate 21, various biochemical tests were performed. Biochemical tests showed that isolate 21 was gram negative rods, motile, catalase and oxidase positive and can also utilize nitrate in respiration. These characteristics point to the *Pseudomonas spp*. Blasting at NCBI GenBank returned three *Pseudomonas aeruginosa* strains with 92% identity. *Pseudomonas aeruginosa* has been previously reported to degrade Isoproturon (Dwivedi *et al.*, 2011), which is a phenylurea herbicide like Diuron.

The opd gene, which encodes Organophosphate Hydrolase enzyme, is a plasmid-borne gene which has been found to display significant genetic diversity. However, the region on the plasmid housing the opd gene is relatively conserved. The puh A gene, which codes for Phenylurea Hydrolase enzyme, has also been found to be located on a plasmid (Sorensen *et. al.*, 2008). The efficiency of hydrolysis of different organophosphate pesticides by Organophosphate Hydrolase (OPH) enzymes from different bacteria differs considerably. This has been shown to be due to base substitutions in the opd gene, resulting in OPH enzymes that have different rates of stereoselectivity and substrate specificity due to slightly different conformations of their active sites (Casey *et. al*., 2011; Van Dyk and Brett, 2011). Differences in nucleotides at certain positions are evidence of the molecular variation e.g. positions 1204 and 1205 for isolate 7. Isolate 7 could have taken up a plasmid with opd genes via transformation or conjugation, since the metagenome in the study soil has other bacteria which possess the opd gene. For isolate 21, there are variations in the nucleotide sequences between the isolate and its BLAST top hits e.g. at positions 374-376. Other studies have shown *Pseudomonas aeruginosa* has the ability to degrade diuron due to repeated stress from application of the pesticide, resulting in genetic adaptation via increase in puh A and puh B copy numbers (Egea *et. al.*, 2017)

Chlorpyrifos biodegradation proceeds by oxidative dealkylation, yielding TCP as the main metabolite and diethyl phosphates as minor metabolites. Tiwari et. al. (2014)

reported biodegradation of Chlorpyrifos following first order kinetics, resulting in TCP as the main metabolite. Chen et. al. (2012) reported that both Chlorpyrifos and TCP biodegradation by fungal strain Hu-01 followed first order kinetics. Diuron biodegradation occurs via N-demethylation, which occurs successively, followed by cleavage of the amide bond. The metabolite 3,4 DCA undergoes dechlorination to form aniline and 4-chloroaniline. Deamination of aniline then occurs, giving catechol (Egea *et. al*, 2017). The enzymes that have been found to participate in biodegradation include phenylurea hydrolase and aniline dioxygenase. In the case of Diuron, while a number of soil bacteria have been found to be capable of degrading Diuron, few soil bacteria possess the ability to mineralize 3,4 DCA (Devers-Lamrani *et.al.*,2014). Sorensen et.al. (2008) found that the bacteria *Arthrobacter globiformis* strain D47 was able to degrade Diuron to 3,4 DCA but was not capable of mineralizing the harmful 3,4 DCA metabolite to harmless CO_2 in pure culture. Egea *et. al.* (2017), however, found that P. aeruginosa TD2.3, isolated from soil used in sugarcane farming, was able to degrade both Diuron and the metabolite 3,4-DCA after 5 days, with the initial Diuron concentration being 50mg/L. It was found to degrade 29% of Diuron, with 3,4 DCA being detected at a very small concentration. Since this concentration accounted for only a small fraction of the Diuron degraded, it showed that the P. aeruginosa likely had the ability to completely degrade 3,4 DCA. This agrees with the results of this study. The other Diuron metabolite DCPMU was not detected, which also agrees with the results of this study.

For Chlorpyrifos, the R^2 values obtained for first order degradation equations were positive and may indicate a first order kinetics. However, the values are not as high as 0.99, and therefore in order to qualify the results as first order kinetics, other factors have to be considered. The values are not significantly different, and they range from 0.6504 to 0.8704, and are not proportional to their respective inoculum densities. The first order assumption is further supported with half-lives (less than two days) which are similar for all inoculum densities. For Diuron, R^2 values obtained for first order degradation equations ranged from 0.885 to 0.984, and again are not proportional to their respective inoculum densities. Although the values are close to 0.99, none of them are as high as 0.99, and the half-lives at every inoculum density were between 8 and 16 days. The length of the half-lives for Diuron do not support first-order kinetics. For each

initial concentration, the fit to a first order model is very close with R^2 values close to 0.99. If the rate constants and half-lives differ considerably, that indicates deviations from first order kinetics. It is evident that predictions based on first order kinetics model are not reliable if half- lives are in the range of 11 – 35 days (Richter *et. al.*, 1996). Non-linearity of the first order degradation kinetics is difficult to analyze from a single experiment, according to the Michaelis-Menten kinetics. Unless the initial concentrations are high above saturation, pseudo zero order cannot be distinguished from first order kinetics due to measurement errors (Richter *et. al.*, 1996). Typically, degradation of a pesticide is a biotic process. However, there are abiotic degradation processes such as photodegradation and also various types of chemical degradation processes. Assuming negligible abiotic degradation processes such as chemical degradation, pesticide degradation is then taken to be an enzyme catalyzed transformation and the rate of an enzyme-catalyzed reaction can be described by Michaelis-Menten kinetics. Michaelis-Menten kinetics is applicable to a situation in which the microbial cells participating in the degradation are not growing to any appreciable degree. If this is the case and if C << Km (i.e., C is very low), then first-order kinetics are consistent with Michaelis-Menten kinetics and degradation may be described by the first-order model. From the R^2 values, the First Order kinetic model is best suited to explaining biodegradation at all but one of the inoculum densities for Chlorpyrifos with *Kosakonia oryzae*, and at all the inoculum densities for Diuron with *Pseudomonas aeruginosa*. From these results, it can be inferred that the density of degrading bacteria and their associated enzymes is large relative to the number of pesticide molecules.

Generally, several bacterial and fungal strains resident in soil have the potential to degrade Chlorpyrifos and Diuron. The molecular and biochemical biodegradative potentials of these species and strains differ with respect to the pesticides and metabolites in question. Some microorganisms are able to degrade both the pesticide and metabolite, while others only degrade the pesticide, resulting in accumulation of the metabolite. The aim of bioremediation is complete mineralization of the harmful xenomolecule to harmless products. The bacteria isolated in this study were found

capable of degrading their respective pesticides and metabolites under laboratory conditions.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study established that a bacterial strain of *Kosakonia oryzae* resident in soils used in sugar cultivation in Western Kenya has the genetic and biochemical traits required to degrade the pesticide Chlorpyrifos and TCP. To our knowledge, this is the first instance that the biodegradation of an organophosphate pesticide by a strain of *kosakonia oryzae* has been demonstrated. The study also isolated a strain of *Pseudomonas aeruginosa* and demonstrated its ability to degrade Diuron, 3,4- DCA and DCPMU. For this study, it was established that:

1. Two bacterial isolates capable of degrading pesticides were isolated from sample soils by Enrichment Culture Technique.
2. 16 S rRNA analysis was successfully used to identify two bacterial isolates which had the ability to degrade pesticides. A bacterial isolate highly similar to *Kosakonia oryzae* (95 % similarity) was found to have the ability to degrade Chlorpyrifos. Another bacterial isolate highly similar to *Pseudomonas aeruginosa* (92% similarity) was found to have the ability to degrade Diuron.
3. Varying the inoculum density of pesticide-degrading bacteria has a non-linear effect on the extent of biodegradation of the respective pesticides and their metabolites. Out of three kinetic models (First, Second and Third Order) the First Order Model best described the degradation of both Chlorpyrifos and Diuron. However, it did not completely fit the biodegradation data for the conditions of this work.

6.2 RECOMMENDATIONS

The following are the recommendations from this study:

1. After further confirmation of their biodegradative abilities, the isolated strains of *Kosakonia oryzae* and *Pseudomonas aeruginosa* from this study should be

further investigated for bioremediation under field conditions as a step in the process of developing biodegradation protocols in-situ.

2. The ability of the *Kosakonia oryzae* strain to degrade Chlorpyrifos suggests the presence of an opd gene in the isolate. As such, this isolate likely has the ability to degrade other organophosphate pesticides. The ability of this isolate to degrade other organophosphate pesticides should be investigated. The ability of *Pseudomonas aeruginosa* to degrade other phenylurea herbicides should also be investigated
3. Bacterial inoculum density as a factor affecting biodegradation should be further studied for these isolates, including monitoring growth of the bacteria using the pesticides and metabolites as substrates separately. Other factors affecting growth and biodegradative activity of these isolates, e.g. pH and temperature, should also be investigated.

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LIST OF APPENDICES

Appendix A: Chlorpyrifos Kinetic Data

Average Chlorpyrifos and TCP concentrations at different inoculum densities at different time interval

Days	Average Chlorpyrifos and TCP levels (mg/L)									
	Inoculum Density 1.5×10^8		Inoculum Density 3.0×10^8		Inoculum Density 6.0×10^8		Inoculum Density 9.0×10^8		Inoculum Density 12×10^8	
	CPF	TCP	CPF	TCP	CPF	TCP	CPF	TCP	CPF	TCP
0	10.14	0.0	10.14	0.0	10.14	0.0	10.14	0.0	10.14	0.0
2	0.64	0.72	0.26	0.171	0.25	0.089	0.22	0.15	0.23	0.12
4	0.45	0.36	0.23	0.1592	0.24	0.0826	0.24	0.14	0.24	0.05
6	0.25	0.25	0.21	0.1570	0.03	0.0814	0.00	0.09	0.00	0.05
7	0.20	0.23	0.22	0.1352	0.00	0.0792	0.06	0.05	0.04	0.03
8	0.01	0.2123	0.01	0.1335	0	0.011	0	0.01	0	0.007

Natural logarithms of concentration of Chlorpyrifos at different inoculum densities (Day 0-Day 8)

Time (Days)	In (Concentration)				
	1.5×10^8 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	2.317	2.317	2.317	2.317	2.317
2	-0.431	-1.347	-1.386	-1.514	-1.470
4	-0.799	-1.470	-1.427	-1.427	-1.427
6	-1.386	-1.561	-3.507	-	-
7	-1.609	-1.514	-	-2.813	-3.219
8	-2.303	-2.303	-	-	-

Natural logarithms of concentration of Chlorpyrifos at inoculum density 1.5×10^8 CFU/ml at different time intervals (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	ln (Concentration)
10.14	0	2.317
0.65	2	-0.431
0.45	4	-0.799
0.25	6	-1.386
0.20	7	-1.609
0.1	8	-2.303

Natural logarithms of concentration of Chlorpyrifos at inoculum density 3.0×10^8 CFU/ml at different time intervals (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	ln (Concentration)
10.14	0	2.317
0.26	2	-1.347
0.23	4	-1.470
0.21	6	-1.561
0.22	7	-1.514
0.1	8	-2.303

Natural logarithms of concentration of Chlorpyrifos at inoculum density 6.0×10^8 CFU/ml at different time intervals (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	ln (Concentration)
10.14	0	2.317
0.25	2	-1.386
0.24	4	-1.427
0.03	6	-3.507
0.00	7	-
0	8	-

Natural logarithms of concentration of Chlorpyrifos at inoculum density 9.0×10^8 CFU/ml at different time intervals (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	ln (Concentration)
10.14	0	2.317
0.22	2	-1.514
0.24	4	-1.427
0.00	6	-
0.06	7	-2.813
0	8	-

Natural logarithms of concentration of Chlorpyrifos at inoculum density 12.0×10^8 CFU/ml at different time intervals (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	ln (Concentration)
10.14	0	2.317
0.23	2	-1.470
0.24	4	-1.427
0.00	6	-
0.04	7	-3.219
0	8	-

Reciprocal of concentration of concentration of Chlorpyrifos at different inoculum densities (Day 0-Day 8)

Time (Days)	1/(Concentration)				
	1.5×10^8 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	0.099	0.099	0.099	0.099	0.099
2	1.539	3.846	4	4.546	4.348
4	2.222	4.348	4.167	4.167	4.167
6	4	4.762	33.33	-	-
7	5	4.546	-	16.667	25
8	10	10	-	-	-

Reciprocal of concentration of concentration of Chlorpyrifos at inoculum density 1.5×10^8 CFU/ml (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	1/(Concentration)
10.14	0	0.099
0.65	2	1.539
0.45	4	2.222
0.25	6	4
0.20	7	5
0.1	8	10

Reciprocal of concentration of concentration of Chlorpyrifos at inoculum density 3.0×10^8 CFU/ml (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	1/ (Concentration)
10.14	0	0.099
0.26	2	3.846
0.23	4	4.348
0.21	6	4.762
0.22	7	4.546
0.1	8	10

Reciprocal of concentration of concentration of Chlorpyrifos at inoculum density 6.0×10^8 CFU/ml (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	1/(Concentration)
10.14	0	0.099
0.25	2	4
0.24	4	4.167
0.03	6	33.33
0.00	7	-
0	8	-

Reciprocal of concentration of concentration of Chlorpyrifos at inoculum density 9.0×10^8 CFU/ml (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	1/(Concentration)
10.14	0	0.099
0.22	2	4.546
0.24	4	4.167
0.00	6	-
0.06	7	16.667
0	8	-

Reciprocal of concentration of concentration of Chlorpyrifos at inoculum density 12.0×10^8 CFU/ml (Day 0-Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	1/(Concentration)
10.14	0	0.099
0.23	2	4.348
0.24	4	4.167
0.00	6	-
0.04	7	25
0	8	-

Inverse of squares of concentration of Chlorpyrifos at different inoculum densities (Day 0- Day 8)

Time (Days)	$1/(\text{Concentration})^2$				
	1.5×10^8 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	0.009726	0.009726	0.009726	0.009726	0.009726
2	2.364066	14.70588	15.87302	20.83333	18.86792
4	4.926108	18.86792	17.24138	17.24138	17.24138
6	15.87302	22.72727	1000	-	-
7	25	20.83333	-	277.78	625
8	100	100	-	-	-

Inverse of squares of concentration of Chlorpyrifos at inoculum density1.5×10^8 CFU/ml (Day 0- Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	$(Concentration)^2$	$1/(Concentration)^2$
10.14	0	102.82	0.009726
0.65	2	0.423	2.364066
0.45	4	0.203	4.926108
0.25	6	0.063	15.87302
0.20	7	0.04	25
0.1	8	0.01	100

Inverse of squares of concentration of Chlorpyrifos at inoculum density 3.0×10^8 CFU/ml (Day 0- Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	$(Concentration)^2$	$1/(Concentration)^2$
10.14	0	102.82	0.009726
0.26	2	0.068	14.70588
0.23	4	0.053	18.86792
0.21	6	0.044	22.72727
0.22	7	0.048	20.83333
0.1	8	0.01	100

Inverse of squares of concentration of Chlorpyrifos at inoculum density 6.0×10^8 CFU/ml (Day 0- Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
10.14	0	102.82	0.009726
0.25	2	0.063	15.87302
0.24	4	0.058	17.24138
0.03	6	0.001	1000
0.00	7	0.0	-
0.00	8	0.0	-

Inverse of squares of concentration of Chlorpyrifos at inoculum density 9.0×10^8 CFU/ml (Day 0- Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
10.14	0	102.82	0.009726
0.22	2	0.048	20.83333
0.24	4	0.058	17.24138
0.00	6	0.00	-
0.06	7	0.0036	277.78
0.00	8	0.0	-

Inverse of squares of concentration of Chlorpyrifos at inoculum density 12.0×10^{8} CFU/ml (Day 0- Day 8)

Concentration of Chlorpyrifos (mg/L)	Time (Days)	(Concentration)2	1/(Concentration)2
10.14	0	102.82	0.009726
0.23	2	0.053	18.86792
0.24	4	0.058	17.24138
0.00	6	0.00	-
0.04	7	0.0016	625
0.00	8	0.0	-

Appendix B: Diuron Kinetic Data

Average Diuron, 3,4 DCA and DCPMU concentrations at different inoculum densities at different time intervals

Days	Inoculum Density 1.5×10^8			Inoculum Density 3.0×10^8			Inoculum Density 6.0×10^8			Inoculum Density 9.0×10^8			Inoculum Density 12×10^8		
	Diuron	3,4 DCA	DCPMU	Diuron	3,4 DCA	DCPMU	Diuron	3,4 DCA	DCPMU	Diuron	3,4 DCA	DCPMU	Diuron	3,4 DCA	DCPMU
0	25.23	0.0	0.0	25.23	0.0	0.0	25.23	0.0	0.0	25.23	0.0	0.0	25.23	0.0	0.0
4	16.83	0.0	0.0	20.14	0.0	0.0	18.69	0.0	0.0	16.04	0.0	0.0	17.43	0.0	0.0
8	15.53	0.0	0.0	12.29	0.0	0.0	13.06	0.0	0.0	12.43	0.0	0.0	15.77	0.0	0.0
12	14.90	0.0	0.0	10.28	0.0	0.0	12.21	0.0	0.0	10.62	0.0	0.0	11.62	0.0	0.0
16	6.69	0.0	0.0	6.08	0.0	0.0	4.56	0.0	0.0	8.18	0.0	0.0	8.27	0.0	0.0
18	5.98	0.0	0.0	5.75	0.0	0.0	4.14	0.0	0.0	6.75	0.0	0.0	7.26	0.0	0.0

Natural logarithm of concentration of Diuron at different inoculum densities (Day 0-Day 18)

Time (Days)	In (Concentration)				
	1.5×10^8 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	3.22803376	3.22803376	3.22803376	3.22803376	3.22803376
4	2.82316301	3.00270789	2.92798862	2.7750856	2.85819286
8	2.74277364	2.50878592	2.56955412	2.52011291	2.7581094
12	2.70136121	2.33020026	2.50225529	2.36273902	2.45272775
16	1.90061387	1.8050047	1.51732262	2.10169215	2.11263451
18	1.78842057	1.74919985	1.42069579	1.9095425	1.98237983

Natural logarithms of concentration of Diuron at inoculum density 1.5×10^8 CFU/ml at different time intervals (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	ln (Concentration)
25.23	0	3.22803376
16.83	4	2.82316301
15.53	8	2.74277364
14.9	12	2.70136121
6.69	16	1.90061387
5.98	18	1.78842057

Natural logarithms of concentration of Diuron at inoculum density 3.0×10^8 CFU/ml at different time intervals (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	ln (Concentration)
25.23	0	3.22803376
20.14	4	3.00270789
12.29	8	2.50878592
10.28	12	2.33020026
6.08	16	1.8050047
5.75	18	1.74919985

Natural logarithms of concentration of Diuron at inoculum density 6.0×10^8 CFU/ml at different time intervals (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	ln (Concentration)
25.23	0	3.22803376
18.69	4	2.92798862
13.06	8	2.56955412
12.21	12	2.50225529
4.56	16	1.51732262
4.14	18	1.42069579

Natural logarithms of concentration of Diuron at inoculum density 9.0×10^8 CFU/ml at different time intervals (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	ln (Concentration)
25.23	0	3.22803376
16.04	4	2.7750856
12.43	8	2.52011291
10.62	12	2.36273902
8.18	16	2.10169215
6.75	18	1.9095425

Natural logarithms of concentration of Diuron at inoculum density 12.0×10^8 CFU/ml at different time intervals (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	ln (Concentration)
25.23	0	3.22803376
17.43	4	2.85819286
15.77	8	2.75810940
11.62	12	2.45272775
8.27	16	2.11263451
7.26	18	1.98237983

Reciprocal of concentration of concentration of Diuron at different inoculum densities (Day 0-Day 18)

Time (Days)	1/(Concentration)				
	1.5×108 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	0.03963535	0.03963535	0.03963535	0.03963535	0.03963535
4	0.05941771	0.04965243	0.05350455	0.06234414	0.05737235
8	0.0643915	0.08136697	0.07656968	0.08045052	0.06341154
12	0.06711409	0.09727626	0.08190008	0.09416196	0.08605852
16	0.14947683	0.16447368	0.21929825	0.12224939	0.12091898
18	0.16722408	0.17391304	0.24154589	0.14814815	0.13774105

Reciprocal of concentration of concentration of Diuron at inoculum density 1.5×10^8 CFU/ml (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	1/ (Concentration)
25.23	0	0.03963535
16.83	4	0.05941771
15.53	8	0.0643915
14.9	12	0.06711409
6.69	16	0.14947683
5.98	18	0.16722408

Reciprocal of concentration of concentration of Diuron at inoculum density 3.0×10^8 CFU/ml (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	1/ (Concentration)
25.23	0	0.03963535
20.14	4	0.04965243
12.29	8	0.08136697
10.28	12	0.09727626
6.08	16	0.16447368
5.75	18	0.17391304

Reciprocal of concentration of concentration of Diuron at inoculum density 6.0×10^{8} CFU/ml (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	1/ (Concentration)
25.23	0	0.03963535
20.14	4	0.05350455
12.29	8	0.07656968
10.28	12	0.08190008
6.08	16	0.21929825
5.75	18	0.24154589

Reciprocal of concentration of concentration of Diuron at inoculum density 9.0×108 CFU/ml (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	1/ (Concentration)
25.23	0	0.03963535
16.04	4	0.06234414
12.43	8	0.08045052
10.62	12	0.09416196
8.18	16	0.12224939
6.75	18	0.14814815

Reciprocal of concentration of concentration of Diuron at inoculum density 12.0×10^8 CFU/ml (Day 0-Day 18)

Concentration of Diuron (mg/L)	Time (Days)	1/ (Concentration)
25.23	0	0.03963535
17.43	4	0.05737235
15.77	8	0.06341154
11.62	12	0.08605852
8.27	16	0.12091898
7.26	18	0.13774105

Inverse of squares of concentration of Diuron at different inoculum densities (Day 0- Day 18)

Time (Days)	$1/(\text{Concentration})^2$				
	1.5×108 CFU/ml	3.0×10^8 CFU/ml	6.0×10^8 CFU/ml	9.0×10^8 CFU/ml	12.0×10^8 CFU/ml
0	0.00157096	0.00157096	0.00157096	0.00157096	0.00157096
4	0.00353046	0.00246536	0.00286274	0.00388679	0.00329159
8	0.00414627	0.00662058	0.00586292	0.00647229	0.00402102
12	0.0045043	0.00946267	0.00670762	0.00886647	0.00740607
316	0.02234332	0.02705159	0.04809172	0.01494491	0.0146214
18	0.02796389	0.03024575	0.05834442	0.02194787	0.0189726

Inverse of squares of concentration of Diuron at inoculum density 1.5×10^8 CFU/ml (Day 0- Day 18)

Concentration of Diuron (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
25.23	0	636.5529	0.00157096
16.83	4	283.2489	0.00353046
15.53	8	241.1809	0.00414627
14.9	12	222.01	0.0045043
6.69	16	44.7561	0.02234332
5.98	18	35.7604	0.02796389

Inverse of squares of concentration of Diuron at inoculum density 3.0×10^8 CFU/ml (Day 0- Day 18)

Concentration of Diuron (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
25.23	0	636.5529	0.00157096
20.14	4	405.6196	0.00246536
12.29	8	151.0441	0.00662058
10.28	12	105.6784	0.00946267
6.08	16	36.9664	0.02705159
5.75	18	33.0625	0.03024575

Inverse of squares of concentration of Diuron at inoculum density 6.0×10^8 CFU/ml (Day 0- Day 18)

Concentration of Diuron (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
25.23	0	636.5529	0.00157096
18.69	4	349.3161	0.00286274
13.06	8	170.5636	0.00586292
12.21	12	149.0841	0.00670762
4.56	16	20.7936	0.04809172
4.14	18	17.1396	0.05834442

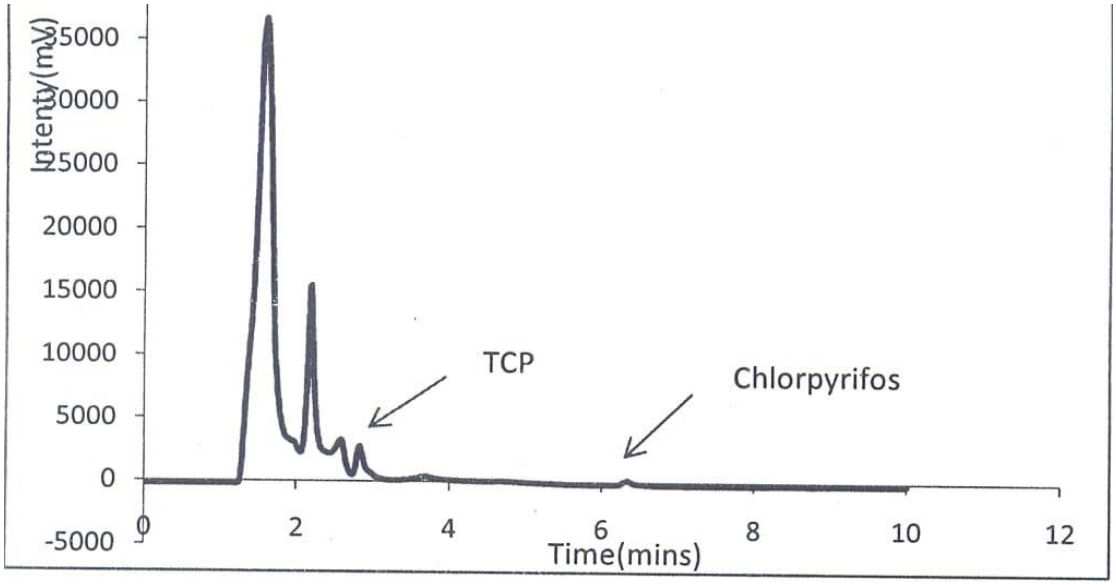
Inverse of squares of concentration of Diuron at inoculum density 9.0×10^8 CFU/ml (Day 0- Day 18)

Concentration of Diuron (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
25.23	0	636.5529	0.00157096
16.04	4	257.2816	0.00388679
12.43	8	154.5049	0.00647229
10.62	12	112.7844	0.00886647
8.18	16	66.9124	0.01494491
6.75	18	45.5625	0.02194787

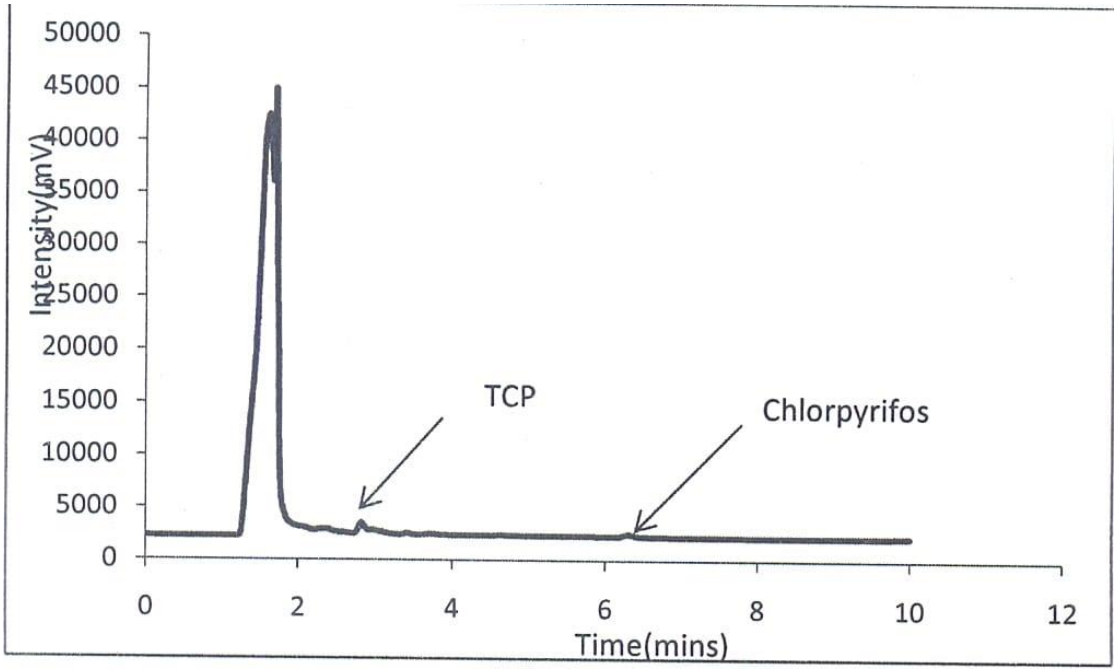
Inverse of squares of concentration of Diuron at inoculum density 12.0×10^{8} CFU/ml (Day 0- Day 18)

Concentration of Diuron (mg/L)	Time (Days)	$(\text{Concentration})^2$	$1/(\text{Concentration})^2$
25.23	0	636.5529	0.00157096
17.43	4	303.8049	0.00329159
15.77	8	248.6929	0.00402102
11.62	12	135.0244	0.00740607
8.27	16	68.3929	0.0146214
7.26	18	52.7076	0.0189726

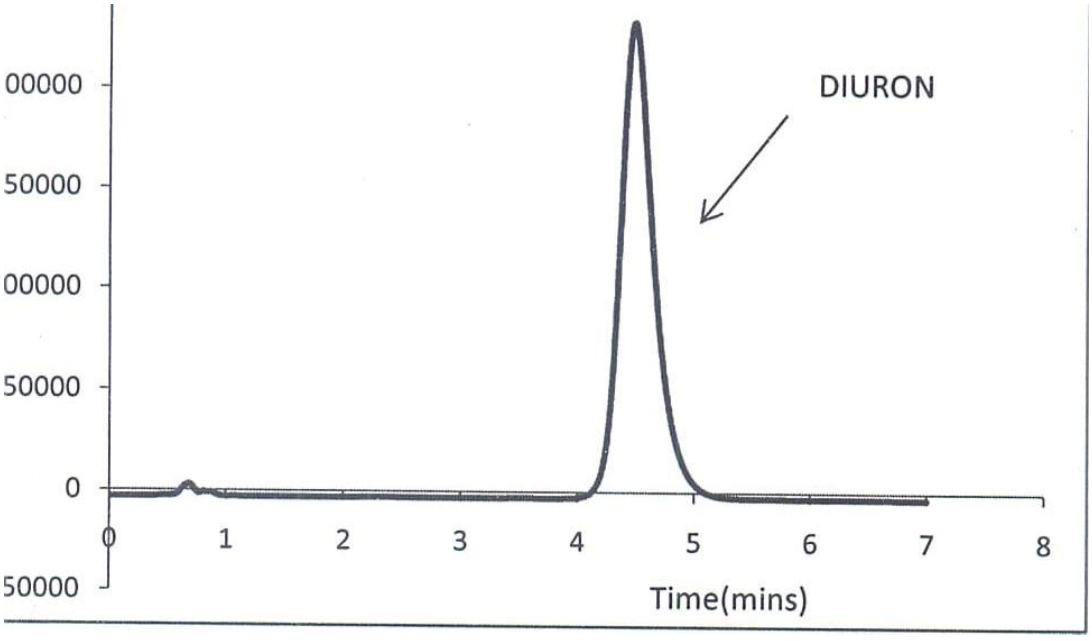
Appendix C: High Performance Liquid Chromatography Chromatograms



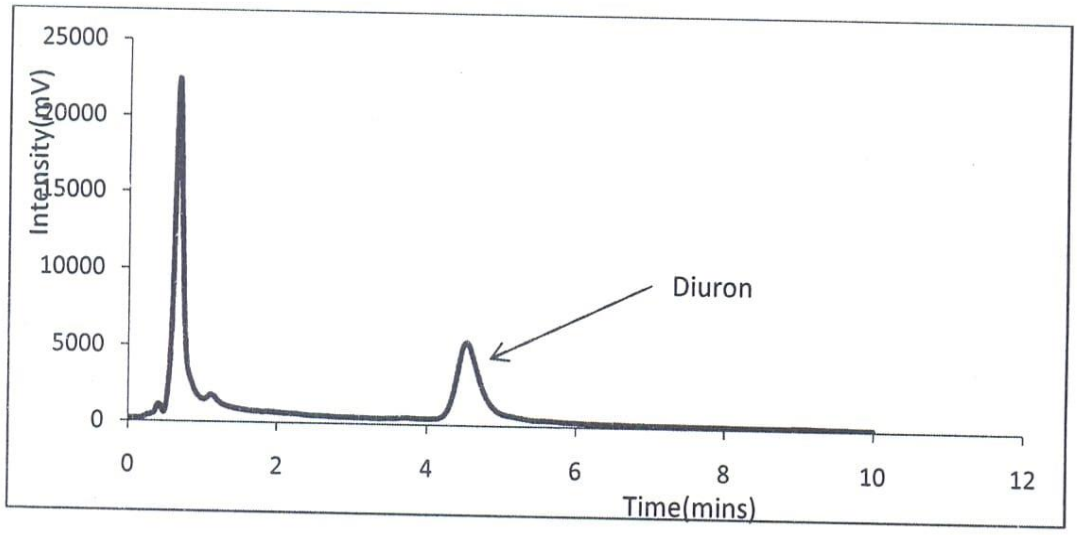
HPLC chromatogram for Chlorpyrifos and TCP for inoculum density 6.0×10^8 CFU/ml after 7 days



HPLC chromatogram for Chlorpyrifos and TCP for inoculum density 12.0×10^8 CFU/ml after 8 days

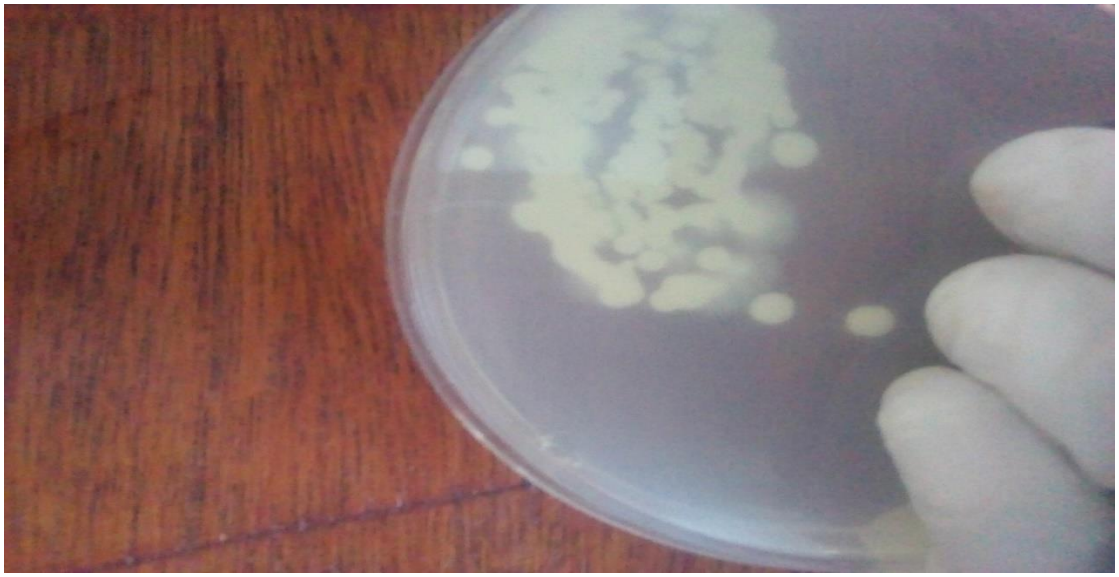


HPLC chromatogram for Diuron Standard (>99% purity)



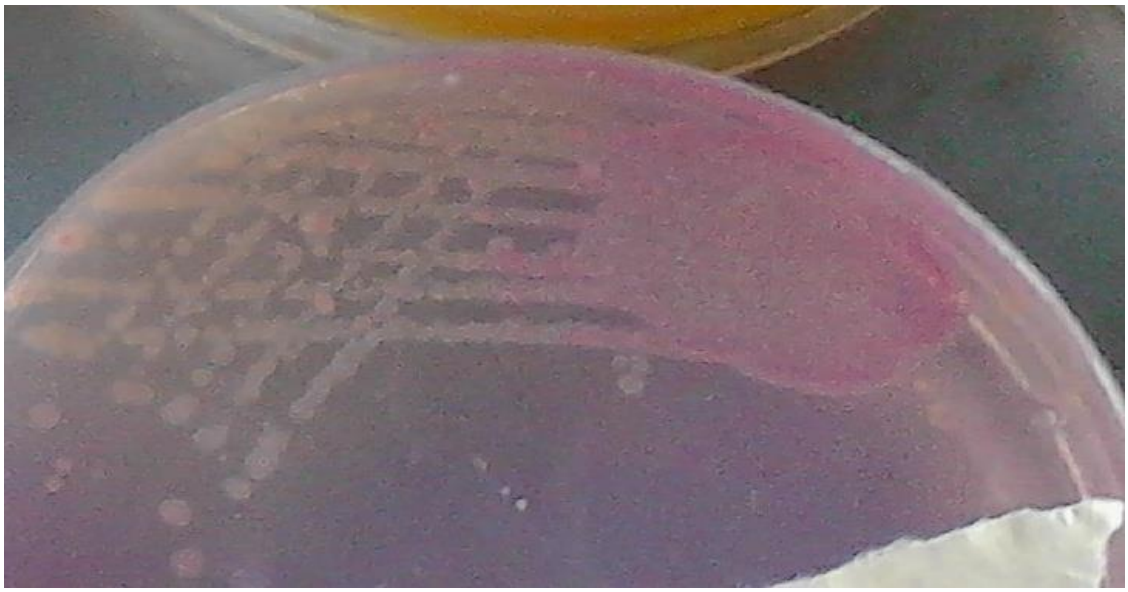
HPLC chromatogram for Diuron for inoculum density 6.0×10^{8} CFU/ml after 18 days

Appendix D: Plates for Results of Selected Biochemical Tests for Bacterial Isolates



Isolate 7 on CPF Mineral Salt Agar

Isolate 21 on Nutrient Agar



Isolate 21 on MacConkey Agar

Triple Sugar Iron Test (Lactose, Glucose and Sucrose) for the isolates: Isolate 7 (Right) and Isolate 21 (Left)

Citrate Test for Isolate 7 (Right) and Isolate 21 (Left)

Voges-Proskauer Test: Isolate 7 (Right) and Isolate 21(Left)

Appendix E: Bacterial Genomic DNA Isolation Protocol

Procedure

1. Add 1.8 ml of microbial culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x g for 30 s at room temperature. Decant the supernatant and spin the tubes again at 10,000 x g for 30 s at room temperature. Completely remove the supernatant with a pipette tip. Note: Depending on the type of microbial culture, it may be necessary to centrifuge longer than 30 s. This step concentrates and pellets the microbial cells. It is important to pellet the cells completely and remove all the culture media in this step.

2. Resuspend the cell pellet in 300 µl of PowerBead Solution and gently vortex to mix. Transfer resuspended cells to a PowerBead Tube. Note: The PowerBead Solution contains salts and a buffer that stabilizes and homogeneously disperses the microbial cells prior to lysis.

3. Add 50 µl of Solution SL to the PowerBead Tube. Note: To increase yields, to minimize DNA shearing or for difficult cells, refer to the Troubleshooting Guide. Solution SL contains SDS and other disruption agents required for cell lysis. In addition to aiding in cell lysis, SDS also breaks down fatty acids and lipids associated with the cell membrane of several organisms. SDS may precipitate when cold but heating at 55°C will dissolve the SDS. Solution SL can be used while it is still warm.

4. Secure PowerBead Tubes horizontally using the Vortex Adapter (cat. no. 13000-V1-24). Vortex at maximum speed for 10 min. Note: This step creates the combined chemical/mechanical lysis conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process. The vortex action is typically all that is required; however, more robust bead beaters may also be used. In most cases bead beating times may be shorter with other devices but you run the risk of increased DNA shearing. This process is compatible with fast prep machines.

5. Make sure the 2 ml PowerBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at a maximum of 10,000 x g for 30 s at room temperature. Note: The cell debris is sent to the bottom of the tube while DNA remains in the supernatant.

6. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Note: Expect 300–350 µl of supernatant. Volume will vary depending on the size of the cell pellet in Step 1.

7. Add 100 µl of Solution IRS to the supernatant and vortex for 5 s. Incubate at 4°C for 5 min.

8. Centrifuge the tubes at 10,000 x g for 1 min at room temperature. Note: Solution IRS contains a reagent to precipitate non-DNA organic and inorganic material, including cell

debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

9. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Note: Expect 450 µl of supernatant. The pellet at this point contains non-DNA organic and inorganic materials, including cell debris and proteins. For the best DNA quality and yield, avoid transferring any of the pellet.

10. Add 900 µl of Solution SB to the supernatant and vortex for 5 s. Note: Solution SB is a highly concentrated salt solution. It sets up the high-salt condition necessary to bind DNA to the MB Spin Column membrane in the following step.

11. Load about 700 µl into a MB Spin Column and centrifuge at 10,000 x g for 30 s at room temperature. Discard the flow-through, add the remaining supernatant to the MB Spin Column, and centrifuge again at 10,000 x g for 30 s at room temperature. Note: Each sample processed will require 2–3 loads. Discard all flow-through. DNA is selectively bound to the MB Spin Column silica membrane. Contaminants pass through the filter membrane, leaving only the DNA bound.

12. Add 300 µl of Solution CB and centrifuge at 10,000 x g for 30 s at room temperature. Note: Solution CB is an ethanol-based wash solution used to further clean the DNA bound to the MB Spin Column silica filter membrane. This wash solution removes residues of salt and other contaminants but allows the DNA to stay bound to the silica membrane.

13. Discard the flow-through. Centrifuge at 10,000 x g for 1 min at room temperature. Note: The flow-through is waste, containing ethanol wash solution and contaminants that did not bind to the MB Spin Column membrane. This step removes any residual Solution CB (ethanol wash solution). It is critical to remove all traces of Solution CB because it can interfere with downstream DNA applications.

14. Place the MB Spin Column in a new 2 ml Collection Tube (provided). Note: Be careful not to splash any of the liquid on the MB Spin Column.

15. Add 50 µl of Solution EB to the center of the white filter membrane. Note: Placing the Solution EB (elution buffer) in the center of the small white membrane will make sure the entire membrane is wet. This will result in more efficient release of bound DNA.

16. Centrifuge at 10,000 x g for 30 s at room temperature. Note: As Solution EB passes through the silica membrane, DNA is released and flows through the membrane and into the Collection Tube. The DNA is released because it can only bind to the MB Spin Column membrane in the presence of salt. Solution EB is 10 mM Tris pH 8 and does not contain salt.

17. Discard the MB Spin Column. The DNA is now ready for downstream applications