

**EFFECTS OF HALOXYFYMETHYLESTER+DICLORVOS (DDVP) ON
NITRIFYING BACTERIA**

BY

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SEPTEMBER, 2023.

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**DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES,
UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA IN PARTIAL
FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF
SCIENCE (B.SC.) DEGREE IN MICROBIOLOGY**

SEPTEMBER, 2023.

CERTIFICATION

We certify that this project work was carried out by **Ofuani Oluchi Gloria (Miss)** in partial fulfilment of the requirement for the award of Bachelor of Science (B.Sc.) degree in Microbiology.

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(Supervisor)

Date

Professor (Mrs.) F. I. Akinnibosun
(Head of Department)

Date

DEDICATION

This project is dedicated to the Almighty GOD, my helper and also to my lovely parents Mr. and Mrs. Ofuani. May they live to reap the fruits of their labour.

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I wish to express my unending gratitude first to the Head of Department, Department of Microbiology, Professor (Mrs) F. I. Akinnibosun and my supervisor Mr A. Dunkwu-okafor for their unreserved support and encouragement. I also wish to thank all of my friends for their unending support throughout my stay in the University of Benin, Benin City.

I would also like to express my gratitude to my family.

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ABSTRACT

Modern agriculture and industries are dependent on a variety of synthetic chemical compounds, including pesticides. Their extensive exploitation has resulted in the contamination of natural environment. This study investigates the effects of the combination these two chemicals, Haloxyfop-R-methyl ester + Dichlorvos, on nitrifying bacteria populations, specifically *Nitrosomonas sp* and *Nitrobacter sp*, within soil samples. Random collection of soil samples was collected from the University of Benin farmland sprayed with the appropriate dosage of the combined chemicals. The soil samples were obtained from four points. This research spanned a 28-day period, with sampling conducted at day 0, day 14, and day 28. Isolation of *Nitrosomonas sp* and *Nitrobacter sp* using Winogradsky medium 1 and 2 involved culturing diluted soil samples in Winogradsky 1 and 2 media. The study results showed a *Nitrosomonas* count of $3 \times 10^3 \pm 424.26$ and *Nitrobacter* count of $8.6 \times 10^3 \pm 2262.74$. The study showed a gradual reduction in *Nitrosomonas* and *Nitrobacter* counts compared to the actual control group. These findings suggest a suppressive effects of the combination of both chemicals on Nitrifying bacteria. These findings emphasize the importance of understanding the long-term impacts of chemical on essential soil bacteria, as they play a crucial role in ecosystem functioning. The initial increase in *Nitrosomonas* counts on day 0 may be attributed to an adaptative response, but sustained exposure to the contaminants appears to diminish this response, resulting in reduced *Nitrosomonas* populations in contaminated soil. This research contributed valuable insights into the ecological consequences of utilizing the combination of chemicals in nitrifying bacteria populations, thereby offering essential information for informed environmental management practices.

CHAPTER ONE

INTRODUCTION

Modern agriculture and industry are dependent on a variety of synthetic chemical compounds, including pesticides, e.g., zoocides, fungicides, herbicides and others. Their extensive exploitation has resulted in the contamination of natural environment, e.g., natural aquifers which are both major and intermediate receivers of the applied pesticides. While pesticides elicit an acute or chronic toxic effect upon microorganisms, the latter are capable of accumulating, detoxifying and metabolizing these compounds and, additionally, use them as a source of carbon (DeLorenzo *et al.*, 2001). The integrity and efficiency of nitrifying bacteria are paramount to maintaining the balance of nitrogen compounds in both natural and agricultural environments. However, the widespread use of agricultural chemicals and pesticides raises concerns about their potential impact on the delicate populations of these essential microbes. Two such chemicals, Haloxymethylester and DDVP (Dichlorvos), have garnered attention due to their extensive application in pest control and their potential to influence non-target organisms, including nitrifying bacteria.

The detrimental effect of pesticides on the species composition of microorganisms' triggers changes in higher trophic levels. This modification involves both qualitative and quantitative changes. It seems indispensable, therefore, to identify the impact of the mentioned xenobiotics on soil and aquatic microorganisms, and thus on the process of primary production, nutrient circulation and decomposition of matter, in which bacteria serve an important function. In addition, due to the significant role of bacteria in the degradation process of toxic compounds in the natural environment, gaining knowledge on the decomposition of these compounds in pure and mixed cultures is also of key significance, as well as their effects on natural populations of microorganisms.

Pesticides do not necessarily distinguish between pests and other living things. The use of pesticides decreases the general biodiversity in the soil. Pesticides can kill beneficial soil bacteria, earthworms, snails, frogs, birds, and other valuable species. Soil microorganisms play a key role in maintenance of soil structure, transformation and mineralization of organic matter, making nutrients available for plants.

Haloxypop-R-methyl ester is a foliar-applied aryloxyphenoxy-propionate (AOPP) herbicide for the selective control of annual and perennial grasses in broadleaf crops (Zhou *et al.*, 2018). . This compound, haloxypop-p-methyl ester (HPME), is utilized to control both broadleaf and grassy weeds in crops such as corn, sorghum, sugarcane, pineapple, Christmas trees, and other plantations (Olayinka and Ore, 2015). While comprehensive knowledge regarding the metabolic reactions of HPME in plants and animals is limited, haloxypop-p has been identified as an effective metabolite within plants and soil. Notably, this herbicide displays selective action, targeting specific varieties of grassy weeds while minimizing its impact on desired crops. A significant advantage of haloxypop-R-methyl ester lies in its effectiveness against a wide range of grassy weeds. Its mode of action involves inhibiting the synthesis of fatty acids in target plants, ultimately stunting their growth and leading to eventual demise (Peterson *et al.*, 2016). This targeted mechanism allows farmers to manage weeds without causing substantial harm to valuable crops, thereby promoting more robust and productive harvests.

Dichlorvos (2,2- dichlorovinyl dimethyl phosphate; DDVP) is an organophosphate compound with different brand names such as Nuvan, DD Force and Sniper, etc. It is one of the most widely used pesticides for the control of household pests, public health pests, and crops and stored product insects (Celik *et al.*, 2008). Dichlorvos is an insecticide and fumigant with contact, respiratory, and stomach poison on stored product pests (Lotti 2001; Nguegang *et al.*, 2005; Booth *et al.*, 2007; Gbave *et al.*, 2012; Perveen and Khan, 2014). Dichlorvos acts by

inhibiting the enzyme acetylcholinesterase (AChE), which is responsible for breaking down the neurotransmitter acetylcholine, resulting in overstimulation of the nervous system and ultimately paralysis and death of the target pest (Celik *et al.*, 2008)

The identification of nitrifying bacterial strains with biological capacities and metabolic capacities to degrade or utilize pesticides as carbon sources is considered one of the promising approaches to enhancing soil fertility in an ecosystem contaminated with these pollutants. Thus, it is important to examine the response of these organisms to these pesticides so that less toxic and more readily biodegradable pesticides may be developed especially if the current ones in use are toxic, persistence and thus do not meet regulatory requirements in terms of their pollution effects in our environment.

Biological sentinels (Nitrifying bacteria and earthworm) activities in the soil are important for mineral and organic matter cycling in ecological systems, with the nitrogen cycle being crucial for all organisms (Katagi, 2010). Microbial nitrification and earthworm are indicator organisms in the soil that are pointer to soil contamination and pollution. Nitrification is a chemoautotrophic process carried out by two bacterial groups that oxidize ammonium to nitrite and nitrite to nitrate Fenchel *et al.*, (1998).

This process is important to the nitrate content in soil, which is the major source of nitrogen assimilated by higher plants and, thus, of considerable ecological and agricultural importance (Katagi, 2010). Earthworms (*Apporectoda longa*) represent a large fraction of soil living biomass in many temperate ecosystems and play important role in soil functioning.

As ecosystem engineers, they influence organic matter dynamics, soil structure, and microbial community (Katagi, 2010). They actively participate in soil aeration, water infiltration and mixture of soil horizons, and they represent an important source of food for many other organisms like birds or moles Hong *et al.*, (1999). Organochlorine pesticides are

used by farmers without proper training on application and understanding of their toxic effects on the environments. More worrisome is the fact that reports have shown that many of these pesticides readily available to farmers are banned and illegal stockpiles Zhou *et al.*, (2006). Residues of pesticides, upon entering the soil, become toxic to non-target biological sentinels organisms (Chapman, 2002).

Nitrifying bacteria are gram-negative, chemoautotrophic, aerobic bacteria that oxidize ammonia to nitrate in the soil in a process known as nitrification (Willey *et al.*, 2011). The ammonia-oxidizing bacteria convert and the nitrite oxidizing bacteria convert ammonia to nitrate in a twostep process that is dependent on the action of two separate species of nitrifying bacteria, the first stage of ammonia oxidation is carried out mainly by the genera, *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosocytis* and *Nitrosogloea* while in the second stage, nitrite formed is converted to nitrate by the genera, *Nitrobacter*, *Nitrocystis*, *Nitrococcus*, *Nitrospina* and *Nitrospira* (Scragg, 2005). Nitrifying bacteria play a very important role in soil fertility. Nitrogen, which is a common soil nutrient element required in large quantity by plant, is largely made available to plants in the form of nitrate ion by the activities of nitrifying bacteria through the process of nitrification (Bona *et al.*, 2011). Thus, this study is focused on nitrifying bacteria and the effects of chemicals on its growth.

Aim

The aim of this study was to investigate the impact of Haloxyfop-R-methyl ester + Dichlorvos on nitrifying bacteria.

Objectives

The objectives of this study were to

1. identify the *Nitrosomonas* spp. and *Nitrobacter* spp population count within sample farm land

2. determine the heterotrophic bacterial count before application of herbicide (haloxyfop-R-methyl ester+dichlorvos)
3. compare *Nitrosomonas* spp. and *Nitrobacter* spp counts upon application of haloxyfop-R-methyl ester+dichlorvos for a 28-day period

CHAPTER TWO

LITERATURE REVIEW

2.1 Soil and Biodiversity

The soil biota forms an obligatory constituent of terrestrial ecosystems and is known as the biological engine of the earth (Ritz *et al.*, 2004). Soil organisms include a tremendous diversity. This rich species diversity facilitates maximum exploitation of the resources available in the different habitats, at various levels of resolution (Ferris and Tuomisto, 2015). Soil organisms such as earthworm play a significant role in the incorporated variety of ecosystem services or functions such as detoxification of noxious elements. Other functions include nutrient supply for plant growth, water regulation, carbon sequestration, nutrient cycling, support for biodiversity (Turbe *et al.*, 2010).

Soil organisms also provide wider collective benefits such as maintenance of biodiversity above and below the ground, increasing permeation of water for prevention of pollution in water courses, organic waste clearance, degradation of pollutants, and a colossal source of genetic and chemical diversity with many other prospective applications.

Microbial biodiversity includes plant growth promoting rhizobacteria, N₂-fixing cyanobacteria, plant disease suppressive bacteria and fungi, and soil toxicant-degrading microbes that enrich the soil by improving the soil quality, promoting soil health, growth, quality, and productivity of crops (Singh *et al.*, 2011). However, environmental pollution such as acid rain may decrease the soil microbial activity and change the metabolic capability of the soil microbial community (Wang *et al.*, 2014). The soil biota plays a key role in a number of environmental processes that are important in sustenance of terrestrial life and is fundamental within agricultural systems (Brussaard *et al.*, 2007). Escalating functional biodiversity in agricultural ecosystems is a foremost ecological approach to fetch

sustainability to production. Various reports from scientific literature state that for benefit of all agricultural systems, the protection and enrichment of biodiversity and biomass of soil biota is imperative.

2.2 Pesticides

Pesticides are the group of chemical substances which are being used to control and repel the pest population. Pesticides also defined as chemical as well as natural agents applied to control or kill the harsh pests like creatures, organisms causing plant diseases and weeds. It also used to control other living organisms, for example nematodes, arthropods except from insect and vertebrates which destroy our food sources and cause many health problems. Pesticides may be summarized as chemical substances which are used in the broad area of crop to protect from insects, weeds, and pests for nourishing and increasing the yield and efficiency of crop. Besides, these pesticides have other benefits like save man's power, time and high efficiency (Wang *et al.*, 2017). Many studies have documented those plants take up pesticides from soil (Fantke *et al.*, 2013; Florence *et al.*, 2015). According to the United States EPA report, in 2012 the worldwide expenses at the manufacturing stage were 5600 crore dollars. During 2008-2012; herbicides accounted for the top most expenses (45%) trailed by the expenses on insecticides, fungicides and other types of pesticides. The term 'pesticide' is not an innovation. It is being used from the ancient times; Sumerians, Greek, and the Romans were applying diverse chemicals to kill insects including sulphur, mercury, copper and plant extracts. But the consequences were not good because of ancient chemistry and lack of the application strategies.

2.3 Classification of Pesticides

Pesticides can be broadly categorized on the basis of applications, target organism and chemical nature. On the basis of application, pesticides can be grouped as agriculture (used to

protect the crop pest, insects and weeds), public health (used to kill vector which causes diseases) and domestic pesticides (used to kill insects like cockroach, bacteria, protozoa, mice etc.). Based on the target organism pesticides can be categorized as insecticides (chemical which is used to kill the insects), fungicides (chemical which is applied to inhibit or to kill the fungus), herbicides (chemicals which are used to control or kill the weeds), rodenticides (pesticides used to kill the rodents), fumigants (they are gaseous pesticides used to kill or control the pest like bedbug) and insect repellents (which are applied on the skin or cloth to keep insects away from the skin and cloth). On the basis of chemical nature, pesticides can be categorized as organochlorines, organophosphates, carbamates, pyrethroids, phenyl amides (carbanilates, acylalanalides, toluidines and acetamides), phenoxyalkonates, trazines, benzoic acid derivatives, benzonitriles, phtalimide derivatives, dipyrids and miscellaneous category (Jayaraj *et al.*, 2016).

Pesticides can be classified as insecticides, fungicides, herbicides, rodenticides, nematocides, molluscicides and plant growth regulators. Each group is specifically designed to target pests, but they put undesired toxic effects on non-target organisms. (Milanovic *et al.*, 2014). Pesticides are known to be one of the extremely useful and beneficial agents for preventing losses of crops as well as diseases in humans. Based on the action, pesticides can be classified as destroying, repelling and mitigating agents. Insects and pests are getting immune to the commercial pesticides due to over usage. Recently pesticides have been developed which target multiple species (SpeckPlanche *et al.*, 2012). Nowadays, chemical pesticides and insecticides are becoming a dominant agent for eliminating pests. When these chemical pesticides are used in a combination of effective natural enemy than that result in enhanced integrated pest management and act as a comprehensive prophylactic and remedial treatment (Gentz *et al.*, 2010). Non-regulated use of pesticides has led the environment into disastrous consequences. Serious concerns about human health and biodiversity are raising due to

overuse of pesticides (Agrawal *et al.*, 2010). Pesticides are considered to be more water soluble, heat stable and polar which makes it very difficult to reduce their lethal nature. Pesticides are not only toxic to people related to agriculture, but they also cause toxicity in industries and public health work places. Depending upon the target species, pesticides can cause toxicities in natural flora, natural fauna and aquatic life (Rashid *et al.*, 2010).

2.4 Advantages of Pesticides

Pesticides are extensively employed in greatest zones of crop, making to diminish infestations due to the pests and thus protect crops from the possible harvest losses and decrease in product quality (Damalas, 2009). There are big variety of encouraging consequences after use of different types of pesticides. Decreased crop loss subsequent of spraying fungicides is an apparent advantage, nevertheless, few are less apparent either as they happen in the medium or long term, or delicate or minor incremental assistances dispersed over a great zone. The effects are the instant consequences of pesticide usage. Three major consequences of the pesticides include controlling farm pests and plant ailment vectors; controlling vectors of human and livestock ailments and annoyance creatures; and averting or controlling creatures that damage other human actions and constructions. The primary advantages are the result of the pesticide's action; the straight gain anticipated after using them. From the 3 major consequences, 26 primary advantages were identified extending from the safety of recreational surface to protection of human lives. The advantages which are less immediately or apparent and consequences of the primary advantages are known as secondary advantages and 31 secondary advantages were recognized, extending from healthier persons to preserved bio-diversity (Cooper and Dobson, 2007). The interrelation between the harmful effects and the advantages of pesticides remains complicated and not easy to conform forever (Zhang, 2018).

2.4.1 Risk of using pesticides

The exposure to the chemical pesticides is extremely destructive for the flora, fauna and the environment.

2.4.1.1 Human Health

The labours working in the pesticide manufacturing organizations, in fields, assassinating of household pests and green house are mostly affected due to pesticide exposure. The greater chance of exposure in the working area of the pesticides is at the time of production and formulation because in this practice risk is very high. In manufacturing site probability of danger is high as they deal with several hazardous chemicals including pesticides, crude materials and harmful solvents (Gangemi *et al.*, 2016; Grewal *et al.*, 2017). Various types of health problems, including cancer, diabetes mellitus, respiratory disorders, neurological disorders, reproductive (sexual/genital) syndromes and oxidative stress are caused due to the direct exposure, handling of pesticides or pesticide residues present in the food stuffs.

2.4.1.2 Environment

Pesticides could pollute soil, water, grass as well as other flora. In addition to assassinating insects or weeds, pesticides could be poisonous to the other creatures such as birds, fish, useful insects and non-target vegetation. Insecticides are usually the further most intensely poisonous type of pesticides, but herbicides could also pose dangers to the non-target creatures (Mahmood *et al.*, 2016).

2.4.1.3 Diagnosis of Pesticides

Pesticides have been detected by several traditional as well as modern-day techniques including spectrometry, chromatography, immunoassays, electrophoresis, biosensors and

solid phase extraction. Massive research has been done on the analysis of pesticides in the food stuffs and environment (Zhang *et al.*, 2011).

Gas chromatography (GC) is the furthestmost used approach for the diagnosis of the pesticides and this technique is applied for the estimation of non-polar and volatile derivatives. Pesticides can be analysed by GC commonly coupled with a particular detector such as a flame photometric detector, electron capture detector, flame ionization detector and nitrogen phosphorus detector (Mahpishanian *et al.*, 2015). To obtain high sensitivity and selectivity, GC can be coupled with mass spectrometer (MS), called as GC-MS (Tankiewicz and Biziuk, 2018). Distinctive types of detectors are employing to identify distinctive pesticides, with different merits and demerits (Grimalt and Dehouck, 2016). Liquid chromatography (LC) or coupled LC is used with variety of detector like ultra-violet, fluorescence and diode detectors (PérezLemus *et al.*, 2019). High performance LC (HPLC) is an admirable approach to detect broad range of pesticides including polar and heat-unstable pesticides (Bidari *et al.*, 2011). The combination of MS with LC is the utmost sensitive and selective method for the detection of the pesticides.

2.5 Pesticides and Soil Environment

Soil has the centre position for the existence of organisms and ensures their survival, the term soil health and soil environment are used to describe the soil property which holds soil physical, chemical, biological characteristics, those maintain productivity and environment quality which promote the health of plants and animals (Doran, 1994). Soil is a mandatory component for terrestrial environment and is acknowledged as “Biological engine of the earth” (Ritz *et al.*, 2004). Before the era of industrial revolution, i.e., early to mid-1900’s, farming practices were environment-friendly and the connection between agriculture and ecology was very strong. Immediately after this, the ecology and farming linkage was

ignored resulting in high productivity at the cost of the environmental quality. Therefore, the agro-ecosystem safety becomes a daunting challenge and is adversely affected the soil health.

Use of pesticides has become an integral part of our modern life in order to meet the demand of a growing population which is expected to be 10 billion by 2050 (Saravi and Shokrzadeh, 2011). As per an estimate of the last decade nearly \$38 billion was spent on pesticides globally. The major fraction of pesticides accumulated in the soil and further repeated use of pesticides may cause lethal effects. The accumulation of pesticides in organo-mineral components of complex structures greatly influence the processes like mobilization, immobilization, bioavailability and transport (Gevao *et al.*, 2003). The degraded pesticides alter microbial diversity, biochemical reactions and enzymatic activity (Munoz-Leoz *et al.*, 2011). The enzymatic pool of soil comprises of free enzymes, immobilized extracellular enzymes and the enzymes secreted by the microorganism well known as bioindicators of soil health (Hussain *et al.*, 2009). The change in enzymatic activity demonstrates the effect of pesticides on soil biological functions (Romero *et al.*, 2010). Pesticide's channel themselves through various biophysical pathways in soil ecosystems. Animals thriving in soil are always under the threat of various chemicals used in agricultural practices, more specifically the pesticides. It is well established that these xenobiotic products are usually difficult to degrade by soil microbes therefore there is always a chance of their entry to various food chains and food webs resulting bioaccumulation and bio-concentration (Maurya & Malik, 2016). Earthworms' bio-accumulate organic pollutant (Jager *et al.*, 2005), heavy metals (Nahmani *et al.*, 2007) and nanoparticles (Canesi & Prochazkova, 2014) through skin and via soil ingestion. The effect of these pesticides applied to soil has effect on earthworm mortality (Panda & Sahu, 2004), reproduction (Schaefer, 2004), metabolism (Brown *et al.*, 2004) and also enhance the mechanism of bioamplification. Earthworms experience inadvertent toxicity

from terrestrially applied pesticides and this uptake of chemical increases bio-concentration of pesticides in earthworms.

2.6 Pesticide effects on microbial activity in soil

Soil fertility is determined by the presence of sufficient nutrients and also a sufficient number and diversity of soil microflora. Microbial diversity is mostly due to the occurrence of various types of organic substrates in soil. The diverse groups of organisms that are mostly unicellular of prokaryotic or eukaryotic origin include bacteria (eubacteria and archaeobacteria), cyanobacteria, actinomycetes, fungi and algae. These soil microbes perform a variety of activities required for the proper functioning of the soil as a dynamic system. Although pesticides are important, their effects on nontarget organisms are of great concern because this poses a risk to the entire ecological system (Kalia and Gupta 2004). In general, the effects of pesticides on microorganisms will vary depending on the chemical dosage, the properties of the soil and various environmental factors (Ecobichon 1991). Because the application or extensive use of pesticides has led to a rapid decline in the quality of the organic matter in soil it also affects the diversity of the microbial flora and fauna. Because these microbes are involved in various element-recycling and -transformation processes, any change in their number or ratio could potentially prohibit/enhance one or other of the reaction chains important for soil fertility. Pesticides affect nontarget microbes by interfering with vital processes such as respiration, photosynthesis and biosynthetic reactions, as well as cell growth and division and molecular composition (DeLorenzo *et al.* 2001). Initially, the application of pesticide decreases microbe number and activity, but as the chemical persists microbes develop tolerance/resistance and recolonize. Ryan (1999) reported that the conventional practice of fertilizer and pesticide application may affect some groups of organisms in the soil, but the overall effect on the soil community would be small. Gupta *et al.* (2000) reported a negative impact of pesticide application on all soil microbes with a

decrease in the average population of all groups studied in soil samples taken from fields under a rice–wheat cropping system.

2.6.1 Microbial activities

Several soil microbial enzymes are hampered or affected by the application of pesticides to the soil. Soil microbial biomass is affected by an array of factors including the physical and chemical properties of the soil (soil factors), temperature, moisture and pH (soil environmental factors) and the use of chemical fertilizers, pesticides, heavy metals, the addition of organic matter, cultivation and crop rotation, seasonal variation, tillage, etc. (soil management factors). Engelen *et al.* (1998) observed that, on application to soil, Herbogil (dinoterb), a reference herbicide, resulted in the inhibition of biomass-related activities and the stimulation of nitrogen mineralization. They recorded the effects on metabolic parameters as determined by monitoring substrate-induced respiration (SIR) and dehydrogenase activity, as well as carbon and nitrogen mineralization. Variations in the complex metabolic fingerprints were recorded using the Biolog system which demonstrated the inhibition of many catabolic pathways after the application of Herbogil. A laboratory microcosm study to investigate the impact of pesticide (insecticide, herbicide and fungicide) application on the health of paddy field soil showed a decrease in soil dehydrogenase activity with increased pesticide concentrations and toxicity increased in the order insecticide > fungicide > herbicide. However, pesticide application did not produce any significant change in soil protein content, although it did cause an increase in the soil phenol content (Subhani *et al.* 2002). Yao *et al.* (2006) reported that the application of a new pesticide acetamiprid at normal field concentrations (0.5 mg kg⁻¹ dried soil) and at high concentrations (5 and 50 mg kg⁻¹ dried soil) had a strong negative influence on soil respiration and phosphatase activity, however, it enhanced dehydrogenase activity after 2 weeks of application. Pampulha and Oliveira (2006) reported that the herbicide combination 60% bromoxynil þ 3% prosulfuron

induced significant changes in the microbial populations of the soil with a long-lasting negative impact on dehydrogenase activity. Adebayo *et al.* (2007) reported a reduction in fungal, actinomycete and protozoal populations in soil on application of Thiodan (4000 and 8000 mg kg⁻¹) and Karate (6000 and 12000 mg kg⁻¹), although there was a significant increase in the bacterial count.

2.6.2 Bacteria and actinomycetes

The application of bactericides, in particular antibiotics in laboratory, glass house and field studies, decreases the number of soil bacteria. Application of bactericides like oxytetracycline led to growth suppression in soil bacteria (Bossuyt *et al.* 2001). Piotrowska-Seget *et al.* (2008) performed a laboratory study to assess the impact of applying successive doses of oxytetracycline (bactericide) or Captan (fungicide) on microbial biomass and activity. They reported that both oxytetracycline and Captan significantly decreased the numbers of culturable bacteria, although total bacterial biomass was not affected. The study indicated that oxytetracycline or Captan application may negatively affect non-target soil microorganisms and their activities. Soil bacterial and actinomycetal forms are involved in carbon transformation reactions, along with the archaeobacterial methanogens. Franzluebbers *et al.* (1994) observed seasonal changes in soil microbial biomass and mineralizable C and N in continuous wheat, continuous wheat–soybean and wheat–soybean–sorghum systems. Endosulfan and butachlor also inhibit the total methanogenic bacterial population with degree/severity of inhibition being influenced by rate of endo-sulphan application. Butachlor treatment inhibits the methanogenic bacterial population (Kumaraswamy *et al.* 1998). Ibekwe *et al.* (2001) reported variations among Biolog fingerprints showing the severe effect of methyl bromide on heterotrophic microbial activity in the first week of application, as well as a shift in all pesticide treatments to a microbial community dominated by Gram-positive bacterial biomass, as demonstrated by the phospholipid fatty acid profile. However, pesticide-

metabolizing microbes may dominate and overpopulate on pesticide application and thus increase the viable cell count of microbial species. Zhang *et al.* (2008) reported an increase in the number of Gramnegative bacteria on application of the insecticide cypermethrin, which may have acted as a nutrient for the growth of these microbes in the cucumber phyllosphere.

2.6.3 Fungi

Soil fungi have a greater ability to resist the application of pesticides, but the application of fungicides drastically affects their population and hence a variety of mineralization/decomposition processes controlled by them. Moharram *et al.* (1994) performed a laboratory study to show the effect of applying pyrazofos (fungicide), bromoxynil (herbicide) and profenfos (insecticide) on the nitrogen fractions (amino N, peptide-N, ammonia-N, total soluble-N, protein-N, total-N) of six soil fungi. They observed a significant increase in total-N in *Trichoderma harzianum* and *Fusarium solani*, whereas a significant decrease was observed with *Aspergillus niger* and *Penicillium chrysogenum* following the application of pyrazofos. In addition to fungicide, the herbicide bromoxynil caused a significant decrease in total-N in *A. niger*, *F. solani*, *T. harzianum* and *Mucor racemosus*, whereas it significantly increased total-N in *Stachybotrys chartarum*. Similarly, the insecticide profenfos caused a significant reduction in total-N in *P. chrysogenum*. In addition to hyphal molds, soil yeasts are also affected by the application of pesticides. Slavikova and Vadkertiova (2003) reported that the fungicide prochloraz inhibited the growth of the majority of yeast strains, while the insecticide triazamate restricted or inhibited growth in all tested yeast strains. Cryptococcus strains were shown to be most sensitive to pesticides, while *Cystofilobasidium capitatum*, *Debaryomyces occidentalis var. occidentalis* and *Trichosporon cutaneum* strains were most resistant to pesticide application.

2.7 Successful alternative strategies to pesticide use

2.7.1 Use of biopesticides

Biopesticides are included among alternative techniques to amend and decrease the impact of chemical pesticide usage, and form one of the foremost strategies of the Integrated Pest Management (IPM) protocol. These include cultural methods of plant resistance to pests, conservation of natural enemies in the crop and the use of insect pest-control products such as ‘microbial insecticides. The IPM concept partly includes the integrated use of biocontrol agents (microbes, insects, extracts/ compounds) along with the judicious, timely and lesser use of chemical pesticides, as well as wise management of agricultural tools and cropping practices/protocols. Biopesticides, an important part of the biological control system, are products/ processes derived from animals, plants and microorganisms such as bacteria and viruses. The most commonly used biopesticides include *Bacillus thuringiensis*-based products, baculoviruses and botanical extracts such as the use of rotenone, pyrethrin, nicotine and azadirachtin (alkaloid produced by neem). In addition to these a fungus, *Trichoderma*, has been used along with biocontrol agents (*Trichogramma* parasitizes and preys upon pest eggs) and *Bacillus thuringiensis* modification.

2.7.2 Bioremediation of pesticide-contaminated soils/land

Bioremediation utilizes the already available arsenal of pesticide-degrading gene pools coding for a number of enzymes such as oxygenases, hydroxylases, hydrolases and isomerases in native pesticide-resistant vanguard microbial populations of contaminated soils/lands to reclaim the site for sustainable agriculture and crop production (Farrar *et al.* 2002). This spans phytoremediation, mycoremediation, mycorrhizoremediation, etc., depending on the type of organism applied/used (Khan 2007). The choice of microbes, plants, mycorrhiza, mushrooms or their combinations in a remediation effort depends on the extent

of the contamination, the nature of the chemicals present, and the amount of the source available for decontamination (Chowdhury *et al.* 2008). However, the hydrophobic nature of most pesticides is the major obstacle to their uptake by microbes or plants.

2.7.3 Utilization of plant-associated microbes

Plant-associated microorganisms play essential roles in agricultural and food safety, and contribute to environmental equilibrium. Their study has classically been based on cultivation-dependent methods, which often recover only 0.01–10% of direct counts. However, studies based on molecular analysis have estimated 4 4000 species per gram of soil. Most of these microorganisms are probably noncultivable, such as the plant symbiotic arbuscular mycorrhiza endomycorrhizae common in many angiosperms and gymnosperms, or are transiently in a viable but noncultivable state (Montesinos 2003). Hence, it could be suggested that certain plant growthpromoting microbes have fastidious nutritional interdependencies on their supporting microfloral forms, resulting in an inability to culture them in pure forms. These plant growth-promoting or disease-suppressive microbes not only exhibit nutritional interdependency, but also have evolved in a manner that, on co-inoculation, may impart extra benefit(s) on the plant. Dwivedi *et al.* (2009) have reported the positive effect of co-inoculation of various arbuscular mycorrhizal fungi (*Glomus* species) with phenazine and diacetylphloroglucinol positive *Pseudomonas fluorescens*, however, an unknown antifungal metabolite-producing *Alcaligenes faecalis* strain SLHRE425 negatively influenced arbuscular mycorrhiza root colonization. Certain soil bacteria and several actinomycetes are well known to produce a few secondary metabolites having antimicrobial activities (Haas and Keel 2003). Production of these antimicrobial compounds by the producers in biologically active concentrations at localized sites in the environment has a substantial impact on the soil rhizospheric population (including soil probiotic and pathogenic microbes) lacking the potential to produce antibiotics (Thomashow *et al.* 2008).

Moreover, a single microbe may have the potential to produce a variety of, perhaps five or six, different antimicrobial products. These antibiotics may contribute to microbial competitiveness and to the suppression of plant root pathogens. Soil bacteria that exhibit antibiotic production include the genera *Pseudomonas*, *Bacillus*, *Rhizobium* and many more. Among the *Pseudomonas* species inhabiting the rhizosphere, certain strains of fluorescent pseudomonads have received particular attention (Vlami 2008) because of their potential to control seed- and soil-borne pathogenic fungi and oomycetes (Raaijmakers and Weller 2001). Important antimicrobial compounds for which a major contribution to biocontrol has been demonstrated are 2,4-diacetylphloroglucinol, pyoluteorin, phenazines, pyrrolnitrin, cyclic lipopeptides and the volatile hydrogen cyanide (Raaijmakers *et al.* 2006; Loper *et al.* 2007). Some strains, such as *Pseudomonas fluorescens* CHA0 and Pf-5, produce multiple antibiotics with overlapping or different degrees of activity against plant pathogens (Bottiglieri and Keel 2006).

2.8. Effects of Haloxyfymethylester and Dichlorvos (DDVP) on Nitrifying bacteria

Haloxyfymethylester and DDVP are both chemical compounds used as pesticides and insecticides. These chemicals can have adverse effects on various organisms, including nitrifying bacteria like *Nitrosomonas* and *Nitrobacter*, which play a crucial role in the nitrogen cycle in soil and water. Here are some potential effects:

1. Inhibition of Nitrification: Both Haloxyfymethylester and DDVP can inhibit the activity of nitrifying bacteria. *Nitrosomonas* and *Nitrobacter* are responsible for converting ammonia (NH₃) into nitrite (NO₂⁻) and then into nitrate (NO₃⁻). Pesticide exposure can disrupt this process, leading to a buildup of toxic ammonia in the environment.

2. Reduced Nitrogen Cycling: Inhibition of nitrification can disrupt the nitrogen cycle, affecting the availability of essential nutrients for plants and other organisms. This disruption can have cascading effects on ecosystems.

3. Ecotoxicity: Haloxyfymethylester and DDVP can be toxic to a wide range of organisms, including non-target species in soil and water. This toxicity can lead to imbalances in ecosystems and harm to aquatic life and terrestrial organisms.

4. Residue Accumulation: Residues of these pesticides can persist in the environment for extended periods, potentially continuing to affect nitrifying bacteria and other organisms over time.

It's essential to use pesticides like Haloxyfymethylester and DDVP with caution, following recommended application rates and safety guidelines to minimize their impact on beneficial microorganisms and ecosystems. Additionally, the specific effects can vary depending on factors such as concentration, exposure duration, and environmental conditions. Conducting proper risk assessments and considering alternative pest control methods is advisable to mitigate potential harm to nitrifying bacteria and the broader environment.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Collection of samples

Four (4) samples were collected from the University of Benin. The soil samples were gotten from four points using a quadrant. The samples were transported aseptically to Microbiology Laboratory, University of Benin, Benin City for microbiological analysis.

3.2 Isolation of microorganisms from the soil samples:

10g of each soil samples were weighed diluted in 90ml of sterile distilled water. 1ml of the stock solution was further diluted in 9ml of sterile water making a 100-fold dilution. 0.1 ml of the dilution was dispensed into the Petri dishes by pour plate method and allowed to solidify. *Nitrosomonas* was isolated using Winogradsky medium for nitrification phase 1 [(NH₄)₂SO₄, 2.0g; K₂HPO₄, 1.0g; MgSO₄·7H₂O, 0.5g; NaCl, 2.0g FeSO₄·7H₂O, 0.4g; CaCO₃, 0.01g, Agar 15.0g;] Distilled water 1000ml and Winogradsky medium for nitrification phase 2 [(NH₄Cl): 0.5 grams, (KNO₃): 0.5, (MgSO₄): 0.2 grams, (CaCl₂): 0.02 grams, FeSO₄·7H₂O, 0.4g, Agar 15.0g] Distilled water 1000ml. These media were sterilized by autoclaving and aseptically dispersed into sterile Petri dishes after cooling to about 45⁰C. The Petri dishes were then inoculated with and incubated aerobically for 4 days at room temperature (28±2⁰C).

3.3 Preparation and Sterilization of Culture Media

All culture media was prepared according to the manufacturer's instructions. Sterilization will be at 121°C at 15psi for 15 min unless otherwise stated by manufacturer.

3.3.1. Winogradsky medium Phase 1 and 2 media

Fifteen grams (15g) of Winogradsky agar was dissolved in 1000 ml of water. The medium will be placed in an autoclave to sterilize it for 15 minutes at 121 °C. After sterilization, it will be allowed to cool.

3.3.2 Nutrient agar

Twenty-eight grams (28 g) of nutrient agar was dissolved in 1000 ml of distilled water in a conical flask corked with cotton wool and foil paper and allowed to dissolve in 1000 ml of distilled water in a conical flask. The medium will be placed in an autoclave to sterilize it for 15 minutes at 121 °C. After sterilization, the flask will be allowed to cool.

3.4 Enumeration and isolation of total Nitrifying bacterial and count

A 100-fold serial dilution of the samples were prepared aseptically in sterile distilled water. An aliquot of 0.1 ml was inoculated using the pour plating technique. Winogradsky and Nutrient agar was used for enumeration. Plates were incubated at 37±2°C for 24 hours. The number of colony forming unit per millilitre (cfu/ml) was calculated using the formula below:

$$\frac{cfu}{ml} = \frac{\text{number of colonies} \times \text{dilution fold/series}}{\text{volume of inoculum}}$$

(Willey *et al.*, 2008)

3.4.1 Phenotypic identification of Bacteria from samples

Several tests such as Gram reaction, catalase, urease, indole, oxidase, sugar fermentation, citrate utilization, respective reaction on triple sugar iron agar tests were carried out to presumptively identify bacterial isolates (Holt *et al.*, 1994).

3.5 Morphological identification

3.5.1 Gram staining:

This test was done to confirm the cell type of the bacteria to be used. Gram staining techniques was used for differentiation between Gram-positive and Gram-negative bacteria. Organisms that retain the primary stain are called Gram positive while those that do not retain the primary stain when decolourized are called Gram negative. The non-retention of the stain is due to the cell composition. The Gram stain procedure is as follows:

A smear of the bacteria isolate was made on grease free slide and heat fix by passing over flame. The smear was flooded with crystal violet which is the primary stain for 1min then washed with distilled water. Subsequently the slides were flooded with Lugol's iodine solution for 30sec and then washed off with distilled water. 95% alcohol was used for decolorization for 10sec and immediately washed off with distilled water. Finally, the smear was counter stained with safranin for 1min and washed off. The slides were allowed to air dry before observing under the microscope using an oil immersion objective lens of $\times 100$ magnifications to view the slides.

3.6 Biochemical identification

Biochemical test was carried out so as to help in the identification of the bacteria isolates as phenotypic (cultural) characteristics is not sufficient. The various biochemical test carried out are shown below;

3.6.1 Citrate utilization test

This test is used to identify which of the isolate can utilize citrate as the sole source of carbon for metabolism. The medium used for this test is Simon's citrate agar. In the preparation, 22 g of commercially available Simon's citrate agar was dissolved in a litre of distilled water and

sterilized by autoclaving at 121 °C for 15 minutes. The medium is dispensed into test tubes and the test organism was inoculated by stab-inoculating the medium on the tubes using sterile straight inoculation wire containing culture. The tubes were incubated at 37 °C for about 24 hours. Positive result is indicated by a change in colour from green to bright blue colouration.

3.6.2 Sugar fermentation and production of gases using Triple sugar iron agar (TSI)

TSI was prepared following manufacturer's instruction and the prepared media was placed in a test tube and kept in a slant position for it to solidify. The slant and butt of the medium was inoculated with the test bacterium using a sterile loop and it was incubated for 18- 24 hours. The results were read on the basis of acid or alkaline production in the slant or butt region of the tube and gas production was confirmed by the presence of crack or air bubbles in the slant or butt region. More so, production of hydrogen sulphide was confirmed by the blackening of the medium. A prepared laboratory chart was used for result interpretation in line with microbiological standard protocol as well as other biochemical tests carried out on the isolates to confirm or ascertain their identity.

3.7 Data Analysis

The data were analysed using the SPSS package version 21.0. All data are mean of three replicates. The mean, range and standard deviation of each parameter was determined. The means were separated using Duncan's Multiple Range test (SPSS, 2010).

CHAPTER FOUR

RESULTS

The following section presents the results obtained from a comprehensive study examining the impact of a combination of two chemicals (Haloxymethylester + Dichlorvos) on the populations of nitrifying bacteria, specifically *Nitrosomonas* and *Nitrobacter*.

Table 4.1 shows the Cultural, morphological and biochemical characteristics of *Nitrosomonas* and *Nitrobacter* isolates.

Table 4.1: Cultural, morphological and biochemical characteristics of *Nitrosomonas* and *Nitrobacter* isolates

Morphological		
Elevation	Raised	Raised
Margin	Entire	Entire
Colour	Brownish	Cream
Shape	Round	Round
Size	Small	Small
Staining	+	+
Gram stain	-	-
cell type	Rod	Rod
Arrangement	Single	Single
Colour	Pink	pink
Spore staining	-	-
Biochemical		
KOH String Test	+	+
Catalase	+	+
Indole	-	+
Citrate	+	-
Oxidase	-	-
Motility	+	+
Urease	-	-
Glucose	+	+
Sucrose	-	-
Lactose	-	+
Mannitol	-	-
Gas formation	-	+
H ₂ S formation	-	-
TSI (Slant/Butt) reaction	K/K	A/AG
Esculin Hydrolysis	-	-
Identity	<i>Nitrosomonas</i> sp.	<i>Nitrobacter</i> sp.

Key:

K= Alkaline A=Acid

G= Gas

+ = Positive

- = Negative

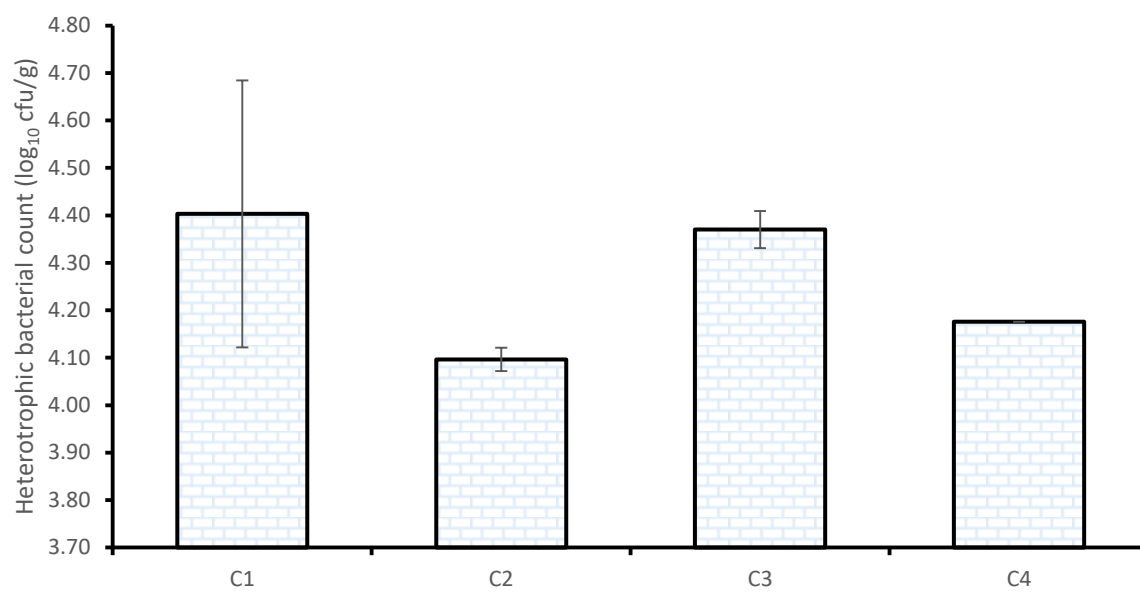


Figure 1.1: Total heterotrophic bacterial count of from soil samples.

Table 4.2: Bacterial Colony Count for *Nitrobacter spp*

Days	Control		Haloxypop-R- methyl ester + Dichlorvos	
	Mean count \pm SD	logcfu/g	Mean count \pm SD	logcfu/g
Day 0	$8.6 \times 10^3 \pm 2262.74$	4.01	$1.095 \times 10^4 \pm 1060.66$	4.07
Day 14	$1.745 \times 10^5 \pm 777.82$	4.23	$1.39 \times 10^4 \pm 282.84$	4.14
Day 28	$2.43 \times 10^4 \pm 3676.96$	4.43	$1.575 \times 10^4 \pm 353.55$	4.20

Table 4.3: Bacteria Colony Count for *Nitrosomonas spp*

Days	Control		Haloxypop-R- methyl ester + Dichlorvos	
	Mean count \pm SD	logcfu/g	Mean count \pm SD	logcfu/g
Day 0	$3 \times 10^3 \pm 424.26$	3.52	$7.15 \times 10^3 \pm 212.3$	3.85
Day 14	$1.165 \times 10^4 \pm 494.97$	4.08	$1.155 \times 10^4 \pm 1767.77$	4.11
Day 28	$2.015 \times 10^4 \pm 1626.35$	4.28	$1.35 \times 10^4 \pm 1131.37$	4.10

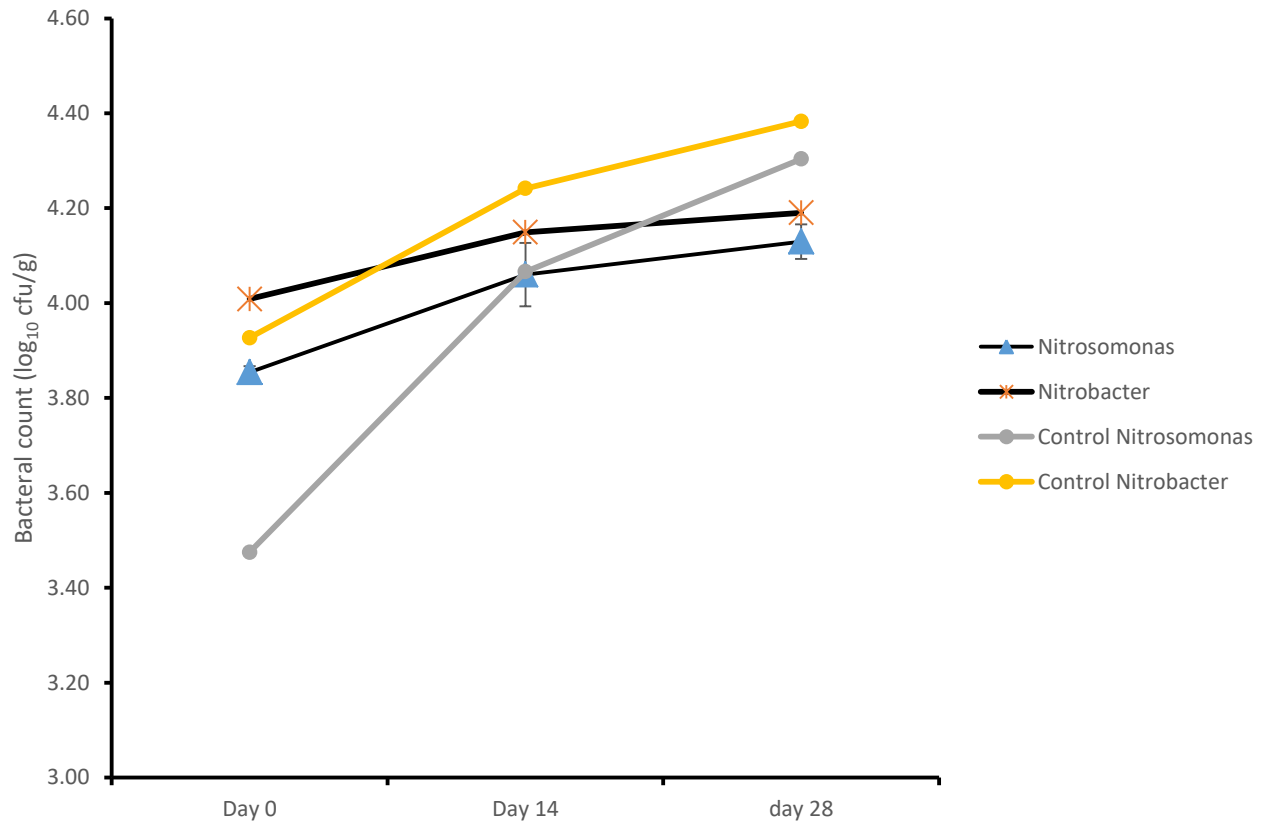


Figure 2.1: Total bacterial count of *Nitrosomonas* and *Nitrobacter*.

CHAPTER FIVE

RESULTS AND DISCUSSION

The effects of Haloxymethylester and DDVP on nitrifying bacteria, specifically *Nitrosomonas* and *Nitrobacter*, were investigated in this research. The study aimed to understand how these chemical contaminants impact the populations of these essential bacteria involved in the nitrogen cycle.

The study found an interesting pattern in the *Nitrosomonas* counts when compared to the control group. On day 0, there was a slight increase in *Nitrosomonas* counts, from 3.47 log₁₀cfu/g for the control to 3.85 log₁₀cfu/g for the contaminated soil samples. This initial increase might be attributed to a temporary response of *Nitrosomonas* to the presence of the contaminants, possibly as a survival mechanism. This results are consistent with (Newman *et al.*, 2016; Aralujo *et al.*,2003) who concluded that the presence of glyphosate decreased the number of bacteria, microbial biomass and acidobacteria population. They believed the reduction in the bacteria population for a long time could weaken some biogeochemical reactions accomplished by these microorganisms.

However, as the study progressed to day 14 and day 28, the *Nitrosomonas* counts in the control group remained relatively stable, with minor fluctuations from 4.07 log₁₀cfu/g to 4.06 log₁₀cfu/g and from 4.30 log₁₀cfu/g to 4.13 log₁₀cfu/g, respectively. On the other hand, the *Nitrobacter* count showed a different trend. The *Nitrobacter* counts in the contaminated samples showed a range of 3.93 log₁₀cfu/g to 3.85 log₁₀cfu/g for day 0, 4.24 log₁₀cfu/g to 4.15 log₁₀cfu/g for day 14, and 4.38 log₁₀cfu/g to 4.19 log₁₀cfu/g for day 28 and results from the pesticide and herbicide contaminated soil were consistently lower than those in the control group. This significant difference in *Nitrosomonas* and *Nitrobacter* counts between

the control and contaminated samples on days 14 and 28 suggests that Haloxyfymethylester and DDVP have a suppressive effect on *Nitrosomonas* populations over time.

Comparing our findings to relevant studies, it's worth noting that our results align with research conducted by Goswami *et al.* (2013), which reported similar suppressive effects of chemical contaminants on *Nitrosomonas* populations in agricultural soils. However, our observation of the initial increase in *Nitrosomonas* counts on day 0 diverges from some earlier studies, such as the work of Anderson *et al.* (2017), which did not observe such an initial response. These discrepancies highlight the complex and context-dependent nature of microbial responses to contaminants, which may vary depending on factors such as soil composition, contaminant concentration, and microbial community composition.

The reason for the initial increase in *Nitrosomonas* counts on day 0 in the contaminated samples could be attributed to a stress response or adaptation of the bacteria to the presence of these contaminants. However, as the exposure continued, it appears that the negative effects of the contaminants started to outweigh any initial adaptive responses, leading to reduced *Nitrosomonas* populations in the contaminated soil compared to the control.

This research highlights the importance of studying the long-term effects of chemical contaminants on crucial soil bacteria like *Nitrosomonas*, as it provides valuable insights into their resilience and potential impacts on the nitrogen cycle, which is vital for ecosystem health and nutrient cycling. Further research could delve into the mechanisms behind this suppression and its ecological consequences.

The results of our plant growth-promoting test involving *Nitrosomonas* and *Nitrobacter* Among the tested strains of *Nitrosomonas*, one strain, designated as *Nitrosomonas A*, exhibited a positive effect on plant growth. This positive result suggests that *Nitrosomonas A* possesses certain characteristics or mechanisms that promote plant growth. Unfortunately, the

two other tested strains of *Nitrosomonas*, designated as *Nitrosomonas B* and *Nitrosomonas C*, did not exhibit any significant positive effects on plant growth. This outcome indicates that not all *Nitrosomonas* strains may possess plant growth-promoting abilities. The absence of a positive effect could be due to variations in strain-specific characteristics, such as the ability to produce growth-promoting metabolites or form beneficial interactions with plants. In addition to the *Nitrosomonas* strains, we also assessed the potential plant growth-promoting capabilities of *Nitrobacter*, another essential nitrifying bacterium. Unfortunately, the *Nitrobacter* strain tested in this study did not demonstrate any discernible positive effects on plant growth. This outcome aligns with the general understanding that *Nitrobacter* primarily participates in the conversion of nitrite to nitrate during nitrification rather than directly promoting plant growth. Grossbard and Atkinson, 1985) reported that the toxic effects of pesticides as result of inhibition of amino acid synthesis via the shikimic acid pathway. Conversely, other studies have demonstrated significant increase in bacteria count after glyphosate treatments (Partoazar *et al.*, 2011; Wardle and Parkinson,1990).

The variations in the plant growth-promoting abilities among different strains of *Nitrosomonas* and *Nitrobacter* highlight the complexity of microbial interactions in agricultural ecosystems. While some strains may positively influence plant growth, others may not exhibit such effects. These results underscore the importance of selecting specific microbial strains carefully for biofertilization or soil inoculation purposes. In practical agriculture, it is crucial to identify and utilize beneficial strains to maximize crop yield and sustainability.

5.1 Conclusion

In conclusion, our journey into the intricate relationship between Haloxyfymethylester, Dichlorvos, and the delicate nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, has revealed a

captivating tale of nature's resilience and vulnerability. As we stand at the threshold of understanding the effects of these chemicals on these microbial heroes of the nitrogen cycle, we are reminded once again of the intricate web that binds all life on Earth.

Our exploration has unveiled a spectrum of responses, ranging from subtle perturbations to more pronounced alterations in nitrifying bacterial populations. Haloxyfymethylester and DDVP, initially designed for pest control, have left their mark on the microbial realm, emphasizing the interconnectedness of all living organisms. These effects, though subtle at times, bear the potential to ripple through ecosystems, influencing the availability of nitrogen, a fundamental building block of life.

Yet, amid the scientific data and laboratory findings, we find solace in the enduring adaptability of *Nitrosomonas* and *Nitrobacter*. These remarkable microbes, guardians of the nitrogen cycle, have demonstrated a resilience that inspires awe. In the face of challenges posed by chemical agents, they persist.

This research calls for a nuanced approach to chemical management practices, one that balances the immediate needs of pest control with the preservation of essential ecological processes. It underscores the importance of sustainable agriculture and environmental stewardship, where the delicate harmony of nature's cycles is both respected and nurtured.

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APPENDIX

Biochemical identification

Oxidase test

Procedure

This is mainly used to differentiate between *Pseudomonas* from other Gram-negative rods. Oxidase test was carried out to identify bacteria species that will produce cytochrome oxidase enzyme. *Staphylococcus aureus* and *Escherichia coli* which are Gram-positive and Gram-negative respectively were employed as control. A piece of filter paper using sterilized wire loop 2-3 drops of freshly prepared oxidase reagent (1% aqueous tetramethyl-3-phenyl nediamine dichloride) was added. A positive oxidase test is indicated by purple colouration within 10 seconds.

Urease test

Procedure

This is used to test organisms that have the ability to produce the enzyme urease which catalyzes the breakdown of urea to produce ammonia. The test is usually used to differentiate organisms like *Proteus mirabilis* from another non-urease positive organism. A sterilized medium was dispensed into test tubes aseptically and the test bacteria isolated were inoculated into the medium and incubated at 37 °C for 24 hours. A change in colour from yellow to red-pink confirmed the presence of urease.

Indole production test

Procedure

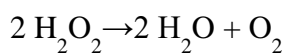
This test was used to determine which of the isolates has the ability to split indole from tryptophan present in peptone water. The test is usually used in differentiating Gram-negative Bacilli especially those of enterobacteriaceae. Five grams of commercially available peptone broth was dissolved in 1litre of distilled water. The medium was then sterilized by autoclaving at 121 degrees centigrade for 15 minutes. The 4 ml of the medium was dispensed

into sterile test tube and each of the bacterial isolates was inoculated into the peptone broth. The inoculated media was incubated at 37 °C for 24 hours after which few drops of KOVAC reagent was added. KOVAC reagents consist of 150 ml of amylalcohol, 10 g dimethylamino benzaldehyde and 150 ml of concentrated hydrochloric acid. Positive test was indicated by the red colouration that occurs immediately at the upper part of the test tube.

Catalase test

Procedure

This is a test to detect the presence or absence of catalase enzyme. The catalase enzyme catalyses the breakdowns of hydrogen peroxide to release free oxygen gas and the formation of water. A few drops of freshly prepared 3% hydrogen peroxide were added onto the bacterial isolates smeared on a slide. The production of gas bubble indicated catalase enzyme positive.



Potassium Hydroxide (KOH) test

Procedure

The potassium hydroxide (KOH) test was used to determine or confirm Gram-negative bacteria, to quickly differentiate between Gram-negative and Gram-positive bacteria as a complement to Gram staining. KOH breaks down the thin peptidoglycan bacterial cell walls of Gram-negative bacteria but does not affect the thick layer of Gram-positive cell walls. Disruption of bacterial cell walls lyses the cell and releases its contents, including the genetic material. A drop of 3% KOH solution was applied on a labelled clean microscope slide and smeared with pure isolated culture using a loop. It was stirred carefully and observed that the solution turned to be a viscous or dense suspension, which formed a slimy or mucoid string within 60 seconds, and the appearance of that indicated a positive result as the presence of Gram-negative isolates. While non-slimy viscous suspensions remained negative results.

Urease test

Procedure

The urease test is used to identify bacteria capable of producing the urease enzyme. The organisms that secrete urease can hydrolyze urea to ammonia and carbon dioxide. This test was used to distinguish urease-positive bacteria from other *Enterobacteriaceae*. The isolated pure bacteria were inoculated into well-prepared and autoclaved Christensen-modified urea broth and incubated for 24 hours at 37°C. Urease-positive cultures produced a pink colour due to a change in the indicator's colour in the presence of ammonia, while the negative result remains no colour change or yellow-orange colour.

Mannitol Test

Procedure

The mannitol test is selective (the presence of high salt concentration; sodium chloride inhibits most Gram-negative and Gram-positive bacteria) and differential test (the ability of the organism to ferment or not the mannitol). The ability to ferment mannitol induces acidification, changing the medium's coloration from red to yellow. A well-prepared mannitol salt agar was autoclaved at 121°C for 15 minutes, cooled and plated. Inoculated with pure isolates and incubated for 24 hours at 37°C. Growths were observed with yellow zones and yellow colonies as positive results. The negative result remained red-pink colour with growths.

Table 3.1: Total heterotrophic bacterial count.

Heterotrophic count (NA)				
Soil ful information	Soil names	Codes	count1	count2
	C1	C1	34	15
	C2	C2	13	20
	C3	C3	21	28
	C4	C4	32	17

Table 3.2: Total *Nitrosomonas* bacterial count (log₁₀cfu/g).

	<i>Nitrosomonas</i>	<i>Nitrobacter</i>
Day 0	3.85421	4.0086002
Day 14	4.060024	4.1492191
day 28	4.12957	4.1903317