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Study On Selective Bacteria Isolated From Pesticide Contaminated Soil To Degrade Glyphosate

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Abstract
The challenge in providing food security for an increasing population requires modern agricultural practices mostly relevant to reducing harvest loses due to diseases caused by pathogens. This investigation was aimed to identify bacteria cells capable of degrading plant herbicide. To achieve this aim isolates with hyper degrading activities were cultured, identified and further more applied for this investigation. The data recorded showed that the following isolates: Rhizobium huautlense strain RA14, Pseudomonas aeruginosa strain MZ4A, Pseudomonas aeruginosa strain 22, Bacillus subtilis strain VBN01 and Pseudomonas aeruginosa strain HS-D38 were identified as most active. The results also showed activities of the isolates in relation to pH and pesticide residue content. Although bacteria exposed to agrochemicals pollutants can adapt to them by mutating or acquiring degradative genes and proliferating in the environment as a result of the selection pressures created by these pollutants, biodegradation may require extremely long periods of time with the adaptation process and reproducibility being very difficult to predict leading to the abuse of pesticide application. The rampant use of these chemicals will cause havoc to humans and other life forms, thus there is a need to explore microbial diversity which can transform toxic pesticides to non-toxic forms.
Keywords: *Bacteria, Pesticides, Toxicity Level, Degradation, Residues*

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I. Introduction

Large volumes of wastes with high pollutants load are discharged into the environment as a result of human activitises. It is estimated that globally more than 1.85 million ha of toxic chemicals are released into the air and water (Hernandez *et al*., 2011). Within the same frame, approximately $6x10^6$ chemicals have been produced, 1000 new chemical products are synthetized annually, and between 60000 and 95000 chemicals are commercially used including synthetic pesticides (Shukla *et al*., 2010). Among those industrial chemicals, pesticides have been identified as significant environmental pollutants (Kontantinou *et al*., 2006; Hildebrandt *et al*., 2008). Pesticide is defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insects, mites, nematodes, weeds, rats, etc.) that has a deleterious effect on crops and results in yield losses (Falkowski *et al*., 2008). Depending on the target-pest, pesticides could be categorized as insecticides, herbicides, fungicides, rodenticides, molluscicides and nematicides (Cerejeira *et al*., 2003). Despite minor variations, the core of pesticide definition remains basically the same: any chemical that is poisonous and efficient against target organisms but entails no risk to non-target organisms and the environment (Zhang *et al*., 2011). Chemical pesticides can be classified according to their chemical composition which are Organochlorine, Organophosphate, carbamates, pyrethroids, Thiocarbamates, Organotin, Organosulfur, Dinitrophenols, Urea derivatives, etc (Falkowski *et al*., 2008). The global agricultural sector is the primary user of pesticides, consuming over 4 million tons annually (Chen *et al*., 2009). It has been increasing annually with 20% of the total world consumption (Abdou and Hend, 2018). Chemical pesticides are still an essential part of modern agriculture and they are extensively used in most cultivated areas aiming to minimize pest infestations, to protect crop yield losses, and to avoid significant deterioration of product quality (Ortiz-Hernandez *et al*., 2013).

Modern agriculture improves crop yields by eliminating or reducing competition from weeds and attacks by pests, safeguards the agricultural products quality, and minimizes labour input (Aktar *et al.*, 2009). Although Pesticides also play an essential role in ensuring reliable supplies of agricultural products each year by preventing fluctuations of annual yields (Cooper and Dobson, 2007; Aktar *et al.*, 2009). On the other hand unregulated use of pesticides has been shown to induce adverse effects to human health and the environment.

The magnitude of those effects depends on the degree of sensitivity of the exposed organisms and the inherent toxicity of the pesticide (Agrawal *et al*., 2010). Risks to human's health by pesticides can occur through direct and indirect exposure. Pesticides can enter the human body through inhalation of aerosols, dust, and vapour that contain pesticides, through oral exposure by consuming food and water, and through dermal exposure by direct contact of pesticides with the skin (Damalas and Eleftherohorinos, 2011; Aktar *et al*., 2009). Apart from human health, the release of pesticide residues in the different environmental compartments including water, air, and soil could result in adverse effects on non-target organisms from all trophic levels including soil microorganisms, beneficial arthropods, plants, aquatics, and birds or mammals (EC, 2006). Previous studies have shown that exposure of soil to pesticide levels substantially higher than their recommended dose could induce inhibitory effects to the structure and the function of soil microorganisms (Karpouzas *et al*., 2013). The development of technologies that guarantee pesticides elimination in a safe, efficient, and economical way is important. Different methods have been developed and implemented to remediate contaminated sites and remove pesticide residues and/or obsolete pesticides (Ortiz-Hernandez *et al*., 2013). However, conventional physicochemical approaches are generally expensive and the remediation process is an alternative for the treatment and detoxification of pesticide-polluted areas (Singh and Walker, 2006).

This technique relies on the ability of microorganisms to convert organic contaminants into simple and non-hazardous compounds. Bioremediation overcomes the limitations of traditional methods for the disposal of hazardous compounds, so it has allowed the environmental removal of many organic contaminants at a reduced cost (Ortiz-Hernandez *et al*., 2013). Thus, bioremediation can offer an efficient and cheap option for the decontamination of polluted ecosystems by pesticides (Singh and Walker, 2006). For these reasons, bioremediation has emerged as a potential alternative to the conventional techniques and could be the most practical method for small-scale companies generating pesticide-contaminated wastes (Felsot *et al*., 2003).

Pesticide Use in Agriculture

Before the development of synthetic pesticides, farmers used naturally-occurring substances such as arsenic and pyrethrum to control pest and diseases. Widespread use of synthetic pesticides began following World War II (Avery, 2006). Pesticide use was credited with increasing crop yields by reducing natural threats to production and became an integral part of agricultural practices by the mid-1950s. Following World War II, pesticides were a component of what was predicted to be a "green revolution" of abundant food for the world. To date the intensive nature of modern agriculture practices has led to the development and wide spread use of synthetic pesticides in our environment (Bhatnagar, 2001). Synthetic organophosphorus compounds have been used worldwide as pesticides, petroleum additives and plasticizers (Zvonko, 2007). These compounds account for more than half of all insecticides used in the world. Statistics have shown that approximately over 5.1 billion of organophosphates are applied to agricultural crops annually (EPA, 2001). Weeds have been a problem in agriculture since about 10,000 BC (Avery, 2006). They have always represented one of the main limiting factors in crop production. Damages globally caused by weeds are responsible for a loss of 13.2% of agriculture production or about $75.6 billion per year (Zvonko, 2007). Weeds represent the most important pest complex since they are relatively constant, whereas outbreaks of insects and disease pathogens are sporadic (Gianessi and Sankula, 2006). Apart from the quantitative damages caused by weeds due to competition with water, light nutrients and to the antagonism (parasitism and allelopathy), weeds are able to cause qualitative indirect damages to crop yield reduction and contamination of seeds (Zvonko, 2007). To overcome the problems caused by weeds, herbicides have largely replaced mechanical methods of weed control in agriculture. Herbicides provide a more effective and economical means of weed control than cultivation, hoeing, and hand pulling. Thousands of urea, also called substituted urea, have been tested as herbicides and many are in use today (Ware and Whitacre, 2004). They include linuron, diuron and monuron, fenuron-TCA, siduron and tebuthiuron. These herbicides can be used as either selective or non-selective weed killer. Their mechanism of action is to inhibit photosynthesis (Donaldson and Kiely, 2002).

Classification of Pesticides

To date about 1000 pesticide formulations or metabolites of pesticides are in use throughout the world (Brar, 2006). Pesticides include all materials that are used to prevent, destroy and repel pest organisms. Insecticides, herbicides, fungicides and rodenticides are some of the more well-known pesticides (Yang *et al.,* 2006). Less well-known pesticides include growth regulators, plant defoliants, surface disinfectants and some swimming pool chemicals. Pesticides are also grouped or classified according to the pests they control, their chemical structure, how or when they work, or their mode of action (Singh *et al.,* 2005). Pesticides with similar structures have similar characteristics and usually have a similar mode of action. Most pesticide active ingredients are either inorganic or organic pesticides. Inorganic pesticides do not contain carbon and are usually derived from mineral ores extracted from the earth. Organic pesticides contain carbon in their chemical structure. Most organic compounds are created from various compounds, but a few are extracted from plant

material and are called botanicals (David *et al*., 2005). Pesticides can also be classified according to how or when they work. Contact pesticides generally control a pest as a result of direct contact, insects are killed when sprayed directly or when they crawl across surfaces treated with a residual contact insecticide. Weed foliage is killed when enough surface area is covered with a contact herbicide. Systemic pesticides are absorbed by pest or plant and move to untreated tissues (Galloway and Handy, 2003). Pesticides can be classified on the basis of how hazardous they are to human beings (EPA, 2009). Insecticides generally are considered the most toxic pesticides to the environment, followed by fungicides and herbicides. However, exceptions exist for certain herbicides which are highly toxic, and are far more hazardous to the environment than are insecticides (Batisson *et al.,* 2007).

Challenges of Pesticide Usage

Ecological effects of pesticides are varied and are often inter-related to each other (USEPA, 2006). Water soluble pesticides are easily transported out of the target area into ground water and streams. Fat soluble chemicals are readily absorbed in insects, fish, and other animals, often resulting in extended persistence in food chains (Batisson *et al.,* 2007). Some of the most troublesome pesticides to the ecology as per World Health Organization classification are (a) insecticides: dieldrin, diazinon, parathion, and aldicarb; (b) herbicides, 2-4-D, atrazine, paraquat, and glyphosate; (c) fungicides: benomyl, captan, mercury, copper, and pentachlorophenol. Organophosphorus compounds have a high index of toxicity and many chronic ecological effects that go often unnoticed by casual observers, yet have great negative consequences in the environment (David *et al*., 2005). They have similar chemical structures and therefore, similar mechanisms of toxicity of inhibiting acetylcholine breakdown in synapses and red blood cell membranes (Cappuccino and Sherman, 2002). These pesticides are highly toxic to target and other non-target animals (Galloway and Handy, 2003).

Approximately three million poisonings and three hundred thousand human deaths occur per year owing to organophosphorus ingestion (Bird *et al.,* 2008). Organophosphate pesticides poisoning resulting in death have also been reported from Kenya (Orme and Kegley, 2006). Acute or chronic toxicity of organophosphorus compounds have also been implicated in a range of nerve and muscular disorders (Ragnarsdottir, 2000). Linuron [3-(3, 4-dichlorophenyl)-1- methoxy-1-methyl urea] is a selective N-methoxy-Nmethyl and it is a substituted phenylurea herbicide that is considered to be moderately to highly toxic to aquatic organisms (Orme and Kegley, 2006). The herbicide is also moderately persistent and relatively immobile in soil, runoff and leaching can result in the migration of the compound to surface- and groundwater bodies, it has frequently been detected in surface and ground waters near or below areas with intensive use, and in one extreme case, linuron was detected in a drinking-water well in concentrations up to 2,800 µg /l (Cappuccino and Sherman, 2002).

Bioremediation of Environmental Pollutants

Bioremediation is a process which utilizes the microbial ability to degrade and/or detoxify chemical substances on sites contaminated with pesticides and other recalcitrant compounds. *In situ* bioremediation exploits the ability of microorganisms to reduce the concentration and/or toxicity of a large number of pollutants on site (Hong *et al.,* 2007). Bioremediation is an economical, versatile, environment-friendly and efficient treatment strategy, and a rapidly developing field of environmental restoration (Rakesh *et al.,* 2005). A well-known example of bioremediation which highlighted the usefulness of this treatment strategy and accelerated its development was the biological clean-up of a large accidental oil spill by the tanker *Exxon Valdez* which ran aground on Bligh reef in the Gulf of Alaska in March 1989, spilling approximately 41,000 m^3 of crude oil and contaminating about 2000 km of sea shore (Yang *et al.,* 2012).

Modern scientists, have created pesticides with chemical structures not found in nature, these unique structures are often responsible for a pesticide's effectiveness and also explains why pesticides can persist in the environment (Abdel- Rasoul *et al.,* 2008). A pesticide's environmental persistence largely depends on its chemical structure and the presence of unusual functional groups, which are large sub-structures within the pesticide molecule. The chemical structure helps to determine its water solubility and consequently, its bioavailability, since microbes more readily assimilate water-soluble compounds (Buyuksonmez *et al.,* 2000). Microorganisms have developed many enzymes that can break down natural compounds, examples are esterase and phosphatase (Jain *et al.,* 2012). Two other classes of enzymes, mono- and dim oxygenases, are also commonly associated with pesticide degradation by introducing one or two oxygen atoms, respectively, into the structure of a pesticide. This oxidation process often makes the pesticide more amenable to further degradation by increasing its water solubility, thereby increasing its bioavailability to the bacteria (Garcinuno *et al.,* 2003).

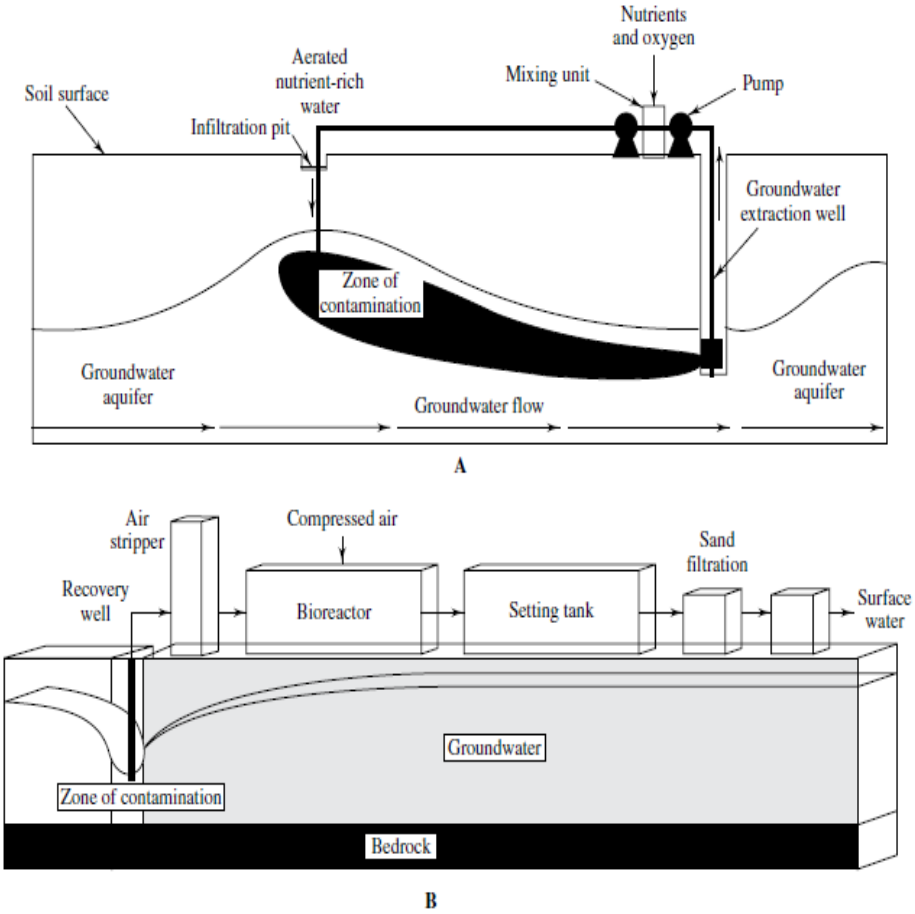

Figure 1: Bioremediation of subsurface contaminated soil and groundwater by (A) *in situ* and (B) *ex situ* bioremediation technology (Hong *et al.*, 2007).

Organophosphate (Op) Pesticides

Organophosphorus pesticides were first developed in Germany by, a chemist Gerhard Schrader in 19th century. Organophosphates are in the form of tetraethyl pyrophosphate and are esters of phosphoric acid which include aliphatic, phenyl and heterocyclic derivatives and have one of the basic building blocks as a part of their complex chemical structure. Currently, organophosphorus group are among the various groups of pesticides that are being used over the world, which is a major and the most widely used group in both domestic and industrial settings (Kanekar *et al.*, 2004).

Organophosphates in Soil

Gundi *et al.* (2004) studied the influence of insecticides on soil microorganisms and their biochemical activities such as nitrification, ammonification, respiration, nitrogen fixation etc. Jain *et al.* (2012) conducted research on the effect of monocrotophos pesticides on fungal strain *Aspergillus niger* in soil of Banasthali region. It was observed that *Aspergillus niger* has the capability of degrading 90% monocrotophos under optimal conditions within 10 days. Lakshmikantha (2000) conducted research on the effect of foliar insecticides fenvalerate, quinalphos and endosulfan on soil microorganisms and their biochemical processes in soils of Gulbarga. Insecticides at normal recommended rate did not affect the major groups of soil microflora *viz*., bacteria, fungi, actinomycetes, free living nitrogen fixers and solubilizers, while four times more than the recommended concentration exerted high depressive effects followed by two times the recommended concentration. Urease and dehydrogenase activities were found sensitive to increased concentration. of insecticides. Pandey and Singh (2002) observed short term inhibitory effects on the total bacterial population after chlorpyriphos and quinalphos application which were recovered within 60 days after seed treatment and by 45 days of soil treatment. The fungal population was significantly enhanced after chlorpyriphos treatment. Singh *et al.* (2002) also observed in a study on effects of chlorpyriphos, fenamiphos and chlorothalonil alone and in combination on soil microbial activity, that the measured soil microbial parameters especially the enzyme activities and total microbial biomass were stable in the pesticide free control soils throughout 90 days of inoculation period, but they were all adversely affected in the presence of added pesticides. De-Lorenzo and

Serrano (2003) analyzed the toxicity of three pesticides, atrazine, chlorpyriphos and chlorothalonil individually and in two mixtures (atrazine and chlorpyriphos, atrazine and chlorothalonil) to the marine phytoplankton *Dunaliella tertiolecta*. At higher concentration of more than or equal to 400 µg/*l*, chlorpyriphos elicited a significant effect on growth rate of *Dunaliella tertiolecta*, while atrazine and chlorpyriphos in mixture displayed additive toxicity (Singh *et al.,* 2002). Menon *et al.* (2004) reported that the arginine ammonification activity of rhizosphere microorganisms were inhibited by chlorpyriphos and its metabolites 3, 5, 6-trichloro-2-pyridinol and 3, 5, 6-trichloro-2- methoxypyridine (TMP) in both loamy sand and sandy loam soils after seed treatment with chlorpyriphos (5 gai/kg). There was also stimulation of rhizospheric N mineralization by parent compound and inhibition by metabolites, whereas it was the reverse in the non-rhizospheric soil. The interaction effects of monocrotophos, quinalphos and cypermethrin when applied either singly or in combination at 0, 5, 10 and 25 µg/ml on microbial population and dehydrogenase activity was studied by Gundi *et al.* (2004). These insecticides significantly enhanced the proliferation of bacteria and fungi, and soil dehydrogenase activity even at the highest level of 25 µg/ml. Also, the antagonistic activities were more pronounced towards soil microflora and dehydrogenase activity when the two insecticides monocrotophos or quinalphos plus cypermethrin were present together in the soil at higher level (25+25 µg/ml), whereas synergistic or additive responses occurred at lower level with the same combination of insecticides in soil. Sardar and Kole (2005) studied metabolism of chlorpyriphos in relation to its effect on the availability of some plant nutrients in soil and observed that there was significant decrease in available nitrogen and phosphorus content in the soil treated with chlorpyriphos at 100 kg ai/ha.

Organophosphates Toxicity

Toxicity of organophosphates to plants, animals and humans has been well documented. The short and long-term exposures to organophosphates induce inhibition of acetyl cholinesterase (AchE) activity, thus leading to nervous impairment in humans and other vertebrates (Monnet-Tschudi *et al.,* 2000). Most of the ill-health following exposure to organophosphorus compounds has been attributed to the inhibition of cholinesterases. However, the current literature has justifiably challenged this view as the inhibition of cholinesterases by itself cannot account for the wide range of disorders that have been reported following organophosphorus poisoning. It is becoming apparent that, although inhibition of cholinesterases plays a key role in the toxicology of organophosphates, individual susceptibility, the inhibition of other enzyme systems and the direct effects of organophosphates on tissues are also important (Monnet-Tschudi *et al.,* 2000). Five organophosphorus pesticides, (Monocrotophos, Omethoate, Parathion-methyl, phoxim and dichlorvos) were examined for their effects on mammalian cell lines to determine their potential impact on physiological functions in vivo (Isoda *et al.,* 2005). Boobis *et al.* (2008) and Bouvier *et al.* (2005) found that exposure to organophosphates is also possible via intentional or unintentional contamination of food sources. Although no clinical effects of chronic, low-level organophosphates exposure from a food source have been shown, advancements in risk assessment and preparedness are ongoing. Organophosphates can be absorbed cutaneously, ingested, inhaled, or injected. Although most patients rapidly become symptomatic, the onset and severity of symptoms depends on the specific compound, amount, route of exposure, and rate of metabolic degradation (Yurumez *et al.,* 2007). Pesticide poisonings are among the most common modes of poisoning fatalities. In countries such as India and Nicaragua, organophosphates are easily accessible and, therefore, a source of both intentional and unintentional poisonings. The incidence of international organophosphate-related human exposures appears to be underestimated (Corriols *et al.,* 2008).

Degradation of Organophosphorus Compounds

Most organophosphorus compounds have a short half-life in the environment, as they are degraded by microorganisms. Enhanced biodegradation of organophosphorus compounds is also influenced by soil properties and the chemical structure of the organophosphorus compounds. Alkaline soils have been shown to be conducive to a higher degradation level of organophosphorous insecticides (Singh *et al.,* 2005). Organophosphorus compounds share similar chemical structures, and therefore soil that developed enhanced degradation for one organophosphorus compound also rapidly degraded other organophosphorus compounds, in a well-known phenomenon called cross-enhanced degradation (Singh *et al*., 2005).

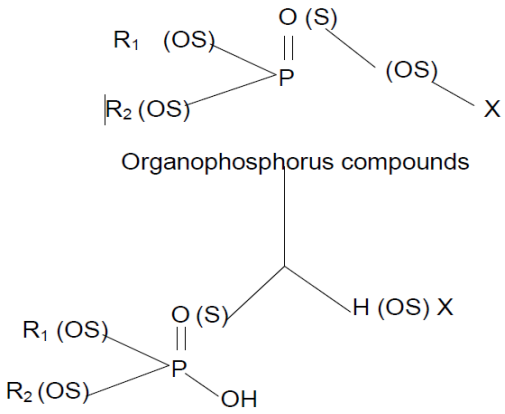

Figure 2: General formula of organophosphorus compounds and major pathway of degradation Source: Sogorb and Vilanova, 2002.

Most organophosphorus compounds are ester or thiol derivatives of phosphoric, phosphonic or phosphoramidic acid. Their general formula is presented in (Figure 2) where 1. R1 and R2 are mainly the aryl or alkyl group, which can be directly attached to a phosphorus atom (phosphinates) or via oxygen (phosphates) or a sulphur atom (phosphothioates). In some cases, R1 is directly bonded with phosphorus and R2 with an oxygen or sulfur atom phosphonates or thion phosphonates. One of these two groups is attached with un-, mono- or di-substituted amino groups in phosphoramidates. The X group can be diverse and may belong to a wide range of aliphatic, aromatic or heterocyclic groups. The X group is also known as a leaving group because on hydrolysis of the ester bond, it is released from phosphorus (Sogorb and Vilanova, 2002).

II. Materials And Methods

Experimental Location and Specimen Processing

The soil samples used were collected from Abeto Farm (6˚34.752'N vand 3˚39.115'E), located at Igbalu, Ikorodu Local Government Area (LGA), Lagos State, Nigeria (Figure 3). Rice and maize are the crops grown in the farm. These rice and maize have been sprayed with glyphosate organophosphorus herbicide for the last 5-6 years. Pesticide contaminated soil samples were collected at four different points in the sampling location, 100 metres apart from each point following sampling procedure described by Asef (2014). The soils were collected using a spatula at a depth of 15cm and transferred to sterile containers. These soils were later placed in plastic bags, transported to the laboratory and stored at 4°C until analysis. Soil samples were air dried and sieved through a 10 mm mesh prior to bacterial screening.

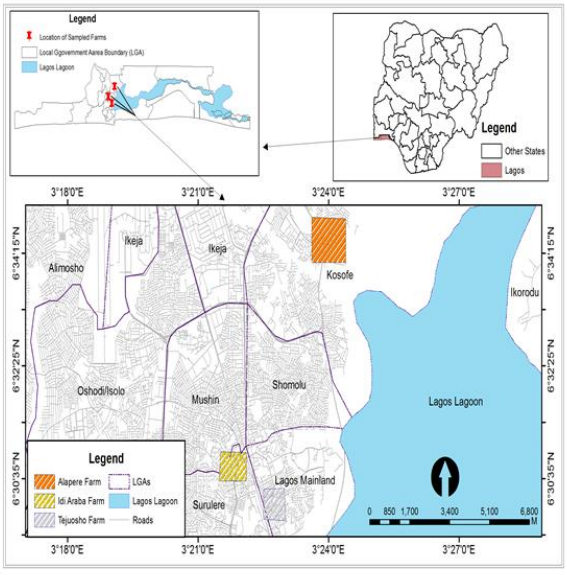

Figure 3: Map showing the sampling location.

Isolation of Organophosphate Degrading Bacteria

Using the contaminated soil, the microorganisms were isolated by serial dilution technique on Minimal Salt Agar Media. In this technique, a sample suspension was prepared by adding 1.0 g sample to 10 ml distilled water and mixed well for 15 minutes and vortexed. Each suspension was serially diluted 10^{-1} to 10^{-7}. 0.1 ml of water was pipetted onto plates with minimal salt media, and then spread with a glass spreader. A sterile filter paper was incorporated with the herbicide and placed on the lid of the plate to create a mobile phase and the plates were incubated at 37°C for 7-14days. After incubation period, the colonies were counted and multiplied by the reciprocal of the dilution factor reported as colony forming unit (Cfu/g). Colonial morphology of the colony was determined and subsequently sub-cultured on three successive occasions using nutrient agar plates. The isolates were maintained on nutrient agar slants after purification and identification of active bateria and kept in a refrigerator for further use (Hiren *et al.,* 2017, Faria *et al.,* 2017).

Screening of Isolates

Screening of Isolates having degradation ability were screened on the basis of degradation of glyphosate. Bacteria which showed higher degradation activity were selected and further examinations were carried out using them. The screening of isolates degrading glyphosate was measured as zones of clearance (Hiren *et al.,* 2017).

Biodegradation Experiment

The ability of the microbial isolates to degrade Force up (Glyphosate) in pure cultures was tested in minimal salt medium which consists g/L $NaHPO_4$, 2.13 g, KH_2PO_4, 1.3 g, NH_4Cl, 0.5 g, $MgSO_4.7H_2O$, 0.3 g and Trace element, 1.0ml. The components were dissolved in 1000ml of distilled water. The pH of the basal medium was adjusted to 7.2 with 1M NaOH solutions. The basal medium of 250 ml Erlenmeyer flasks and the glyphosate was introduced into each flask at 100ppm after sterilization in an autoclave at 121^0C for 15mins. 1.0ml aliquot of diluted broth culture of each isolate (10^4 cells/ml) was seeded into each flask and incubated in a gyratory shaker incubator at 150 revolutions per minute at 30^0C for a period of 32days. (Biodegradation ability) was monitored at fourth day intervals while the (utilization of the pesticide fractions by the microbial isolates) was determined by (monitoring the bacterial growth) determined by viable count using Nutrient agar for the bacteria. The optical density (OD) was measured at 600nm wavelength with PG 70 UV/VIS spectrophotometer and (changes in ionic concentration) (pH) was also determined with pH meter (model p211) (Nwaogu *et al*., 2008).

Quantification of Pesticide Residue

This was carried out using Gas chromatography spectrophotometer on the (biodegraded sample). The aqueous samples were analyzed by directly derivatizing an aliquot and the derivatizing reagent mixture was prepared fresh by mixing one volume of Heptafluoro- butanol to two volumes of Trifluroacetic Anhydride. Aliquots (1.6 ml) of the derivatization reagent mixture were added to 2 ml GC vials. The vials were sealed using screw caps with septa. The vials were then cooled to about -4°C before proceeding. A variable volume of eppendorf tubes were used to add a 36ul aliquot of sample extract (or a dilution of the extracts), or standard solution to the derivatizing reagent. Analyte derivatization was then performed by heating the reaction vials to 95°C for 1 hour, then cooled to room temperature. The excess derivatization reagents were evaporated under a gentle stream of nitrogen. The residue was dissolved in 200 µl of ethyl acetate, capped and saved for GC analysis.

Gas Chromatographic Analysis of Sample Extract

Chromatographic analysis was performed using an Agilent Gas chromatography mass spectrophotometer, equipped with a split/splitless injector and an autosampler. The chromatographic conditions were as follows: HP5-MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness), Helium was used as a gas carrier, with constant flow (1.0 ml min-1) , Injection volume (2 µl (pulsed splitless), Injector temperature was 250ºC, GC temperature program: 50ºC (2 minutes), 30ºC per minute to 160ºC (5 minutes), 5ºC per minute to 180ºC and 10ºC per minute to 270ºC (6 minutes) and the quadrupole mass spectrometer was operated in selective ion monitoring (SIM) mode. The analytes were quantitated using the external standard method with calculations based on peak height. Analyte quantitation was carried out with Agilent Mass-Hunter GC software (Moye and Deyrup 1984.

Biodegradation Efficiency

The percentage loss of the pesticide was calculated as:

$$\frac{\text{pesticide level after contamination} - \text{pesticide level after biodegradation study}}{\text{pesticide level after contamination}} \times 100$$

while the efficiency of biodegradation was calculated using the formulae:
LT - LC
Where LT is % loss of pesticides in treatments and LC is % loss of pesticides in control (Wen-Hsin *et al.,* 2012).

Molecular Identification of active bacteria

Genomic DNA Extraction Protocol according to Trindade (2007). 50-100 mg (wet weight) bacterial cells that have been resuspended in up to 200 µl of water or isotonic buffer (e.g.PBS) or up to 200 mg of tissue were added to a ZR BashingTM Lysis Tube containing 750 µl Lysis Solution. It was secured in a bead fitted with 2 ml tube holder assembly and processed at maximum speed for > 5 minutes. The ZR BashingBeadTM Lysis Tube was centrifuged in a microcentirifuge at > 10,000 x g for 1 minute. 400 µl supernatant was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a collection tube and centrifuged at 7,000 x g for 1 minute.

1,200 µl of Bacterial DNA Binding Buffer was added to the filtrate in the collection Tube. 800 µl of the mixture was transferred to a Zymo-SpinTM IIC Column in a collection tube and centrifuged at 10,000 x g for 1 minute. The Zymo-SpinTM IIC Column has a maximum capacity of 800 µl. The flow was discarded through the Collection Tube and the step 6 repeated. 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin TM IIC Column in new collection tube and centrifuged at 10,000 x g for 1 minute. 500 µl Bacterial DNA Wash Buffer was added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-SpinTM IIC Column was transferred to a clean 1.5 ml micro centrifuge tube and 100 µl (35 ul minimum) DNA Elution Buffer added directly to the column matrix. It was then centrifuged at 10,000 x g for 30 seconds to elute the DNA.

Gel Electrophoresis

The DNA fragments of the bacteria were separated using gel electrophoresis stained with binding-dye. The DNA samples were loaded into wells at one end of a gel, and an electric current was applied to pull them through the gel. The DNA fragments moved towards the electrode. The DNA fragments were seen as bands (Stackebrandt and Goebel 1994).

Polymerase Chain Reaction (Pcr) Analysis

The DNA was subjected to a cocktail mix and condition for the PCR. PCR reaction cocktail consisted of 10 µl of 5 x GoTaq colourless reactions, 3 µl of MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each. The Internal Transcribed Spacer (ITS) gene for characterization of fungi, ITS universal primer set which flank the ITS4, 5.8S and ITS5 region was used;
- ITS4 TCCTCCGCTTATTGACATGS
- ITS5 GGAACTAAAAGTCGTAACAAGG

For bacteria, universal primer set which flank the 16S was used.
- 16SF: GTGCCAGCAGCCGCGCTAA
- 16SR: AGACCCGGGAACGTATTCAC

Polymerase Chain Reaction (PCR) conditions include a cycle of initial denaturation at 94˚C for 5 minutes, followed by 35 cycles of each cycle comprised of 30 seconds denaturation at 94˚C, 30secsonds annealing of primer at 55˚C, 1.5 minutes extension at 72˚C and a final extension for 7minutes at 72˚C (Wawrik *et al.,* 2005).

Purification of Amplified Pcr Product

The amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Sodium acetate 3 M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl eppendorf tube, mixed thoroughly by vortexing and kept at -20°C for at least 30 minutes. Centrifugation was done for 10 minutes at 13000 Revolution per minute and 4°C followed by removal of supernatant (tube was inverted on tissue once) after which the pellet were washed by adding 150 µl of 70% ethanol and mixed, then centrifuged for 15 minutes at 7500g and 4°C. Again all supernatant was removed and tube inverted on paper tissue and allowed to dry in the fume hood at room temperature for 10-15 minutes. It was re-suspended with 20 µl of sterile distilled water and kept in -20˚C prior to sequencing. The purified fragment was checked on a 2% Agarose gel ran on a voltage of 120V for about 1 hour, to confirm the presence of the purified PCR product (Trindade, 2007). The ladder used for bacteria is hyper ladder 1 (figure 1) from Bioloine while 1kbplus ladder from Invitrogen was used for bacteria. The expected base pair of the amplicon is around 850bp. The amplified samples were then sent for sequencing.

Genome Sequencing and Annotation

The amplified fragments were sequenced using a Genetic Analyzer 3130 xl sequencer from Applied Biosystems using manufacturer' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all genetic analysis. The classification/identification of organisms was performed by a local nucleotide BLAST search against the non-redundant version of the NCBI ref database (Tamura *et al.,* 2013).

Phylogenetic Analysis

Phylogenetic relationship analysis was performed on the sequences of isolated microorganisms using Molecular Evolutionary Genetics Analysis version 6 (Tamura *et al.,* 2013). The bacterial isolate sequences were analyzed in order to estimate the occurrence of the isolates during the study. Sequences were prepared using FASTA format and aligned using ClustaW option of the program (Tamura *et al.,* 2013). A phylogenetic dendrogram was constructed based on maximum likelihood with 1000 bootstrap. The resulting dendrogram was interpreted.

Statistical Analysis

The data collected were statistically analyzed using graph pad prism 7.0 while significant means were separated using multiple comparison of two way analysis of variance. Correlation analysis was done to compare the relation between pesticide degradation, colony count, optical density and pH..

III. Results

Bacteria Colony Count for the Different Soil Sample

The bacteria colony count of the soil from the farm is shown in Table 1. Soil collected from point four had the highest microbial load ($33.0X\ 10^6$) followed by soil from point three ($14.0\ X\ 10^6$) and the least was the soil from point one ($2.5X\ 10^6$).

Table 1: Bacterial count of soil collected from the soil types (10^6 cfu)

SOIL TYPE	1 ($x10^6$)	2 ($x10^6$)	3 ($x10^6$)	4 ($x10^6$)
COUNT	2.5±0.5	11.5±0.5	14.0±1.0	33.0±3.0

Soil Glyphosate and Its Metabolite Constituents

The soil glyphosate and Adenosine monophosphate (AMP) constituents differed as shown in table 2 below. It was observed that soil 1 contained the highest organic pollutant (513.3 mg/kg) comprising 319.10mg/kg glyphosate and 194.2 mg/kg AMP. Soil from point three had 329.7 mg/kg glyphosate without AMP. Soil from point two had a total of 58.54 mg/kg comprising 25.57 mg/kg of glyphosate and 32.97 mg/kg AMP. Soil from point four had the lowest with only glyphosate (6.98 mg/kg).

TABLE 2: Glyphosate and its metabolite (AMP) constituents of the farmland (mg/kg)

Name (mg/kg)	Soil 1	Soil 2	Soil 3	Soil 4
Glyphosate	319.1	25.57	329.7	6.98
AMP	194.2	32.97	0	0
Total	513.3	58.54	329.7	6.98

Physiological, Morphological and Biochemical Characteristics of The Pure Culture

The characteristics of the culture media of the various isolates subjected to various test is shown in table 3. The initial screening shows that the bacteria were dominated by isolates with cream colour. Other colours were also observed such as green, yellow, red and orange. Gram reactions shows that they were all gram positive with the exception of isolate K in soil 1, isolate C, D and F in soil 2, isolates i and v in soil 4. All soil 3 isolates were gram +ve. The cellular morphologies of all bacteria were rod in shape except isolate L in soil 1, isolate ii and iv in soil 4 that were cocci in shape. Biochemical test detected the presence of catalase in all isolates, oxidase in some of the isolates. Methyl red were absent in almost all the isolates except isolate G and H of soil 1 and isolates i and iii in soil 4. The growth of isolate on agar and herbicide (for glyphosate utilization) showed poor growth in soil 1, moderate to heavy growth signifying good growth in soil 2, 3 and 4. Generally, the selected five isolates from soil 2, 3 and 4 (1SA- soil2 isolate4D, 1SB- soil2 isolate5E, ISC- soil 3 isolate5Y, ISD- soil4 isolate2ii and ISE- soil4 isolate 4iv) were gram positive except ISA (gram negative); rods in shape except ISD (cocci); catalase positive; oxidase negative except ISC (positive); Methyl Red negative and Voges Prouskauer positive. Their zone of clearance exhibited heavy growth except ISB and ISD which showed moderate growth. Their optimum growth observed in 10% concentration of glyphosate (substrate specificity)

after seven days of test were high ranging from 1.031 in ISA to 0.48 in ISB. Relatively, the selected isolates showed similar cultural characteristics.

Substrate Specificity Test

The isolates were subjected to 7 days of substrate specificity test to determine their optical density (Table 4). The result showed that optical density increased with respect to days. The seventh day had the highest optical density. In soil 1 the highest optical density for day 7 was observed in isolate G (0.261) while the lowest was observed in isolate L(0.116) for the same day. In soil 2, isolate D had the highest optical density (1.031) at day 7 while isolate B had the lowest for the same day(0.098). In soil 3, the highest optical density was recorded in isolate Y (0.806) for day 7 while the lowest was recorded in isolate V (0.086) for the same day. In soil 4, the highest growth for day 7 was recorded in isolate ii (0.852) while the lowest was recorded in isolate v (0.416) for the same day. Generally, soil 1 had the lowest growth while soil 2 had the highest growth. Isolates that were used for biodegradation studies were obtained from soil 2, soil 3 and soil 4.

All alphabets are different isolates from different soils.
A(soil 2 isolate 1), B(soil 2 isolate 2), C(soil 2 isolate 3), D(soil 2 isolate 4), E(soil 2 isolate 5), F(soil 2 isolate 6)
U(soil 3 isolate 1), V(soil 3 isolate 2), W(soil 3 isolate 3), X(soil 3 isolate 4), Y(soil 3 isolate 5), Z(soil 3 isolate 6)
i(soil 4 isolate 1), ii(soil 4 isolate 2), iii(soil 4 isolate 3), iv(soil 4 isolate 4), v(soil 4 isolate 5), vi(soil 4 isolate 6)

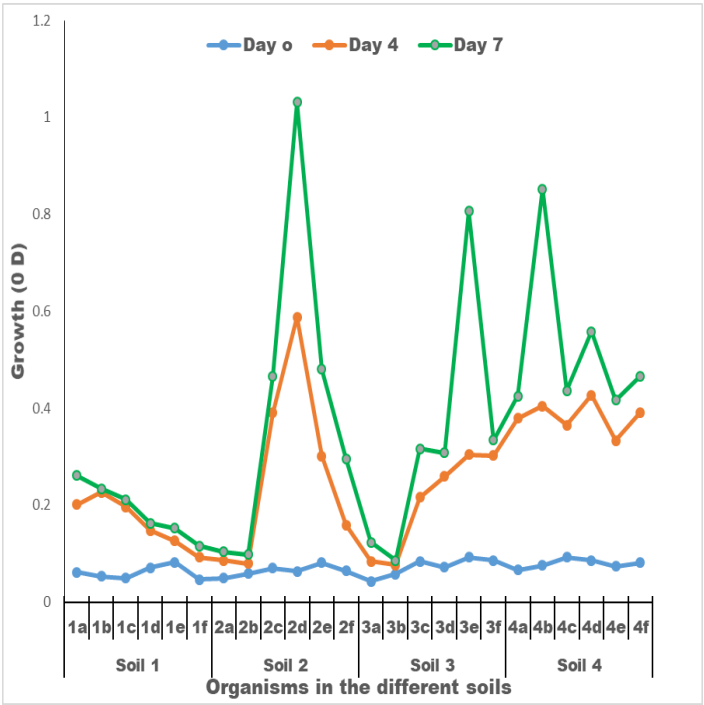

Figure 4: Growth of the different isolates from different soil types represented by optical density

pH of The Culture

The various isolates showed variations in pH within the period of study (Table 5). In isolate A, the day 0 pH was the highest (7.06). It was significantly different from other days ($P < 0.0001$, $P < 0.00001$) except for day 4 which was not significant. All the day 0 isolates had the highest pH in all the culture. Isolate D pH had a significant difference between day 4 and day 0 as well as between day 0 and other days ($P < 0.001$; $P < 0.0001$). Isolate E also had a significant difference between day 0 and day 4 pH as well as between day 0 and other days ($P < 0.05$; $P < 0.0001$). Day 32 had the lowest pH in all the samples. In all, the highest pH was found in isolate B (7.09) while the lowest was in isolate C (5.76). Generally, isolate ISB (*Pseudomonas aeruginosa* strain MZ4A) had the highest reduction in pH (18.48%) while isolate ISA (*Rhizobium huautlense*) and ISE (*Pseudomonas aeruginosa* strain HS-D38) (16.10% each) had the lowest reduction in pH.

IV. Discussion

The intensive use of pesticide to prevent or control pests, including vectors of human or animal disease, unwanted species of plants or animals can lead to an increased risk of contamination of the environment and harmful effects on biodiversity, food security, and water resources) (Queyrel *et al.,* 2016). (Our study has established pesticide contamination of soils in the farm visited). The contaminated soils varied within the different locations on the farmland. It can be as a result of the intensive use of the pesticides for agricultural practices. This is still in agreement with the findings of World Health Organization (WHO) who globally estimated over one million pesticide poisoning cases occurring annually as a result of either long or short term exposure of these pesticides (Azmi and Naqvi, 2011; Tewari *et al*., 2017). Glyphosate was present in all of the soil sampled but their abundance varied. This therefore calls for a remediation action to be taken on the degradation of these Glyphosate to eliminate it from the environment.

Moreover, the microbial abundance/counts varied within the sampled soil. It can attribute to harmful effect of these Glyphosate which has a negative impact on the diversities of microbes. Queyrel *et al.* (2016) has opined that pesticides contribute greatly to biodiversity loss. However, the enumeration of high population of bacteria in the soil samples is an indication that glyphosate could be degraded by the indigenous bacteria isolated from the glyphosate contaminated soil. This is in tandem with the assertion of Manchola and Dussan (2014) and Wokem *et al.* (2017) that enumeration of high population of bacteria is an indication that the organic contaminants can be degraded by the indigenous bacteria in the contaminated soil. This was the basis for the choice of these organisms for their investigation of their biodegradation potentials. Classical microbiology uses both gross and microscopic morphology to identify microbes. Gross morphology includes colony shape, size and surface features (Ritu and Aruna, 2008). The finally selected five isolated strains showed typical morphological and biochemical characteristics of bacteria i.e. gram-positive, rod and cocci-shaped, catalase-positive and oxidase (cytochrome) negative (Ritu and Aruna 2008). It is clear from this study, that the Ikorodu farm of Lagos is dominated by Gram positive and rod shaped bacteria. Moreover, the high growth of the five isolates in the specific herbicide makes them suitable for remediation of glyphosate contaminated environment. Studies have shown that one of the criteria for selecting an organism for remediation of a contaminant is its ability to establish itself fast in the contaminated medium. Therefore this organism may possibly possess the quality for degradation of pesticides.

pH of The Culture

To be able to understand the factors that affect the growth and activity of bacteria for their applications, the influence of pH was analysed. Our study found out that pH levels of culture medium changes with respect to time. This can be as a result of microbial metabolism. It has been found that addition of glucose or other sugars to resting cells of *Serratia marcescens* induced rapid acidification of the extracellular medium. This acidification is due to the catabolism of sugars (Montserrat *et al*., 2000) by bacteria. Analysis of the supernatants on the work of Montserrat *et al*. (2000) demonstrated that the progressive fall in pH resulted from the rapid production of lactic, acetic, pyruvic and citric acids. This is in agreement with our study as there was decrease in pH with respect to time. The implication is that such changes in pH can influence bacteria growth. LeBlanc *et al*. (2004) reported that the growth of *La. fermentum* CRL 722 was noticeably slower at pH 4.5 (μ_{max} = 0.78 h^{-1}) than at other pH values including pH 5.0, 5.5, and 6.0 (μ_{max} = 1.15 − 1.25 h^{-1}).

Optical Density of The Pure Cultures

The study has established that the optical density of the bacteria strains increased continuously during the time interval and the highest OD was reached at the 24th day where it declined till the end of the experiment. This can be attributed to microbial production of secondary metabolites which tend to increase the turbidity of the culture media. Avi-Dor *et al.* (2016) studied the kinetics of the turbidity changes in suspensions of *Pastewella tularertsis*. The initial increase was rapid, caused by the loss of water from the organisms, which decreased in size. This was followed by a decrease in turbidity and the rate of this decrease was affected by compounds which altered the metabolism of the organisms. The study also showed that increase in OD led to a decrease in pH. Francois *et al.* (2005) had similar result. This can be a result of microbial metabolism.

Viable Bacterial Count

Investigation of the bacteria viable count showed variation with respect to time and organism. Bacterial count increased with respect to time for all the isolate. It increased exponentially up to the 32nd day. This can be attributed to environmental factors influencing the relationship with cell count (Francois *et al.* 2005). In general, three distinct phases in the growth of the bacteria could be distinguished, namely, the lag phase, the log phase and the stationary phase. This can be as a result of availability of nutrients leading to constant growth (exponential phase). Yang *et al.* (2018) found that pH level of culture medium was one of the key factors influencing the growth of four bacteriocinogenic strains. They also found that Bacteriocin

production is strongly dependent on medium composition. This clearly indicates the phases of microbial activities. Joseph *et al.* (2001) has linked these phases as a result of turbidity while Yang *et al.* (2018) associated it to pH. This can also suggest their metabolic role toward pesticide degradation. The enumeration of high population of bacteria is an indication that the pesticide sample could be degraded by the indigenous bacteria isolated from the pesticides contaminated soil (Manchola and Dussan, 2014; Wokem *et al.*, 2017). This also implies that the bacteria are able to utilize pesticides as the sole source of carbon and energy (Agostinho *et al.*, 2012). Isolate B having highest count with no significant difference can be the best isolated bacteria for pesticide degradation.

Dna Sequence Analysis/ Molecular Identification of Microbes

Blast analysis of the gene sequence of the pure bacteria culture identified five bacteria species. *Pseudomonas aeruginosa* species, dominated the samples. This relatively high abundance of *Pseudomonas aeruginosa* species in the samples might be due to their high ability to tolerate and degrade pesticides (Darsa, *et al*., 2014). Similar studies have been conducted by Asef (2014) and have documented the isolation, molecular characterization and pesticide degradation by *Aspergillus* species. Therefore, the presence of these bacterial species in our study can suggest their biodegradation potential towards pesticide.

Phylogenetic Analysis

The Phylogenetic analysis revealed that the soil contained diverse bacterial clustering into three orthologous groups. Reason may be the combination of selective factors, proximity and functional capacity of microbes (Ning and Beiko, 2015). Functionally, phylogenetically distant lineages can share common functional features and functions. Ning and Beiko (2015) also opined that functional similarities exist between operational taxonomic units (OTUs) that belong to different high-level taxonomic groups. Most of microbial sequences analyzed in different taxonomic divisions could be related to representatives with known metabolic traits. The study underlines the importance of careful selection of bacteria strains in degradation of glyphosate. *Pseudomonas aeruginosa* strain MZ4A had the highest biodegradation efficiency. This can be attributed to their high viable count and their ability to maintain favorable environmental condition such as pH and OD for degradation of glyphosate. Bacterial consortia for degradation of organic pesticides has been identified with varying success; either showing no positive effect (Arun and Eyini, 2011) or enhanced degradation by specific consortia (Boersma *et al*., 2010; Machín-Ramírez *et al.*, 2010; Lea *et al*., 2014). The last case supports our findings that bacteria may be useful for the remediation of polluting compounds. It is evident that all the strains except *Rhizobium huautlense* strain RA14 can efficiently degrade the glyphosate. The reason may be related to genetic characteristics. This type of studies can provide valuable information to properly develop decontamination procedures to apply *in situ*. Some correlations were also calculated from the results, at the end of the experiment when the organisms were suggested to be highly metabolically active. The negative correlation observed between pesticide degradation and colony count suggests the negative impact pollutants may have on biodiversity. Moreso, viable colony count had a positive correlation with optical density. Dong-ju *et al*. (2012) also observed that bacterial cells showed almost a linear relationship with optical density. These relationships would be useful for biodegradation of glyphosate and other organic contaminants in the environment.

V. Conclusion

In conclusion, from the findings of this study, it suggest that the organisms isolated and identified as *Pseudomonas aeruginosa strain* MZ4A, *Pseudomonas aeruginosa* strain 22, *Bacillus subtilis* and *Pseudomonas aeruginosa* strain HS-D38 have the potential to degrade glyphosate pollutants when applied in the environmentally friendly technology clean-up (bioremediation) of glyphosate contaminated environment. Therefore factors promoting their growth should be encouraged.

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