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Biodegradative Abilities of Indigenous Soil Microflora

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ABSTRACT

Agricultural intensification in Nigeria has dramatically transformed farming practices. Hence, chemical herbicides have become integral to crop management strategies. This study therefore determined the abilities of indigenous microbial consortium to biodegrade herbicides. Treatments of soils with the herbicides were carried out using the complete randomized block design for a period of 8 weeks; at company recommended rates of 4 l/h (at 350 ml in 15 l sprayer), soil treatments were carried out in triplicates. PDA was used for enumeration and isolation of fungi. Incubation of fungal isolates was done at 25^0C for 48 h. The ability of microbial isolates to utilise herbicide substrates in pure cultures were determined in minimal salt medium (g/l) In-vitro biodegradation of herbicides by bacteria and fungi were also determined using gas chromatography. The concentration of residual herbicides in soils after treatment was determined using gas chromatography. The highest optical density value of 1.046 was recorded on xtravest at the 14th day of incubation while gramoxone had the lowest optical density value of 0.885 and 0.955 at the 2nd and 14th days of incubation. *Pseudomonas* spp and *Bacillus* spp occurred frequently in all the herbicide treated soils from the 2nd to the 8th week of treatment. *Actinomyces viscous*, *B. subtilis, P. aeruginosa, P. florescences,* and *P. putida* were also isolated consistently in all the herbicide treated soils. There were significant changes ($P<0.001$) in values of the herbicide concentrations after degradation by bacteria and fungi. This study has shown that indigenous microbial consortium are the best degraders of herbicides. They can be used in the bioremediation of pesticide polluted soils. It also showed that the indigenous microflora could also use the pesticides as nutrient source.

Keywords: Atrazine, Glyphosate, Paraquat, Xtravest

1.0 Introduction

Recently, Nigeria has seen a notable rise in the use of herbicides for weed control (Abakpa *et al*., 2024). The ease of application, efficacy in controlling common weeds in Nigerian farms, and the growing challenge of finding workers to perform the labour intensive manual weed cutting process have all contributed to this increase (Tijani, 2006). However, the quantity of herbicides used in Nigeria increased from 24,935.78 metric tonnes (Mt) to 175,975.49 MT from 2010 to 2020, with a total of 548,327,000,000 MT used during the period based on the Usman (2021) report with data obtained from the National Bureau of Statistics (NBS) Abuja in February 2021 on pesticide imports into Nigeria.

Mechanized farming in Nigeria has dramatically transformed farming practices. Hence, chemical herbicides is frequently used in crop management strategies. Adebayo *et al*. (2022) reported that herbicide usage in Nigerian agricultural system has increased by approximately 67% over the past decade, showing a significant shift in agricultural technological interventions. The environmental implications of uncontrolled herbicide application

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represent a critical research concern. Umar *et al*. (2021) studied the long-term ecological consequences of persistent agricultural chemicals. These herbicides, including glyphosate, atrazine, and 2,4-dichlorophenoxyacetic acid (2,4-D), demonstrate remarkable persistence in soil and water ecosystems, potentially disrupting complex ecological interactions. Microbiological interventions, particularly bacterial bioremediation, have emerged as a promising sustainable approach to managing agricultural chemical contamination. Adekunle *et al*. (2021) emphasized the exceptional metabolic capabilities of specific bacterial genera in degrading complex chemical compounds. Several genera, including *Pseudomonas, Bacillus,* and *Arthrobacter*, have been shown to have high herbicide degrading capacities. These bacteria employ enzymatic systems capable of breaking down complex xenobiotics into less toxic or harmless products, thus facilitating natural attenuation processes in contaminated environments (Putra *et al*., 2024; Zameer *et al*., 2023). In a study by Jayaraj *et al*. (2023), *Pseudomonas nitroreducens* was isolated from agricultural soil and demonstrated high tolerance and degradation capability against multiple pesticides, suggesting its potential application in large-scale bioremediation efforts (Jayaraj *et al*., 2023). Herbicide application does not only reduce bacterial population and diversity, but also shifts microbial community composition in soils. For instance, herbicides such as atrazine and paraquat had been shown to suppress sensitive bacterial species while enriching tolerant genera including *Bacillus* and *Pseudomonas*. Carles *et al*. (2021) reported accelerated degradation of 2,4-D herbicide when co-applied with the degrading strain Cupriavidus necator, reducing herbicide persistence by nearly threefold in agricultural soils (Carles *et al*., 2021). Similarly, engineering multi-degrading bacterial consortia had been shown to significantly improve soil detoxification efficiency under real agricultural conditions (Thieffry *et al*., 2024).

Glyphosate and chloroacetanilide herbicides are the two most widely applied herbicides used extensively in agricultural practices to protect various crops (Choudhury *et al*., 2016; Atwood and Paisley-Jones 2017; Shuman-Goodier *et al*., 2021). Glyphosate is a broad-spectrum, post-emergent, and non-selective systemic herbicide used in various crops such as soybean, cotton, canola, maize, etc. Based on its worldwide usage, the U.S. Environmental Protection Agency has ranked glyphosate as the principal herbicide (Atwood and Paisley-Jones 2017). Asia alone consumes approximately 4.5 × 107 kg of butachlor per year (Kaur and Goyal 2020; Shuman-Goodier *et al*., 2021). Recent reports on herbicides in Indian agriculture state that butachlor in rice, alachlor in groundnut, and glyphosate in cotton and tea are the highest consumed herbicides in India, with an annual consumption of 6,750 metric tons and 6,003 metric tons, (Choudhury *et al*., 2016).

Atrazine, [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine], is a selective herbicide belonging to the family of the s triazines. Due to its high mobility in soil and its massive application, atrazine has often been detected in surface and ground waters at concentrations well above the permissible limits . The moderate persistent, chemical water solubility (33 mg l 1 at 20 ^{O}C) and low soil sorption partition coefficient (Kd ¼ 3.7 L kg 1) are key factors influencing its potential contamination to aquifers, groundwater and rain water as it normally percolates to groundwater or river via infiltration. Atrazine inhibits photosynthesis and its associated noncyclic photophosphorylation is in higher plants. All higher plants probably metabolize atrazine by N-dealkylation with some species such as corn and wheat, which contain benzoxazinone, and utilize hydroxylation as well (Singh and Singh, 2014). Metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-(methoxyprop-2-yl)acetamide) is a selective chloroacetamide herbicide used to control broadleaf and annual grass weeds in corn (*Zea mays* L.), soybean (*Glycine max* L. Merr.), peanut (*Arachis hypogaea* L.) and potato (Solanum tuberosum L.) (Singh and Singh, 2014).

Biodegradation of herbicide treated soils may result in the release of important inorganic matter which plants absorb through their roots into their stems and then to their leaves. Soil fertility is adversely affected by the overuse of herbicides to kill weeds. These therefore leads to reduction in crop production resulting in high cost of food and reduced food supply for both human and animal

consumption (Sharma *et al.,* 2009) The effect of herbicides in the removal of weeds which may lead to increased application could result in reduction in microbial population where the herbicides kill soil microorganism important in breaking down organic matter to inorganic matter hence its uptake by plants (Sharma *et al*., 2019). The excessive use of herbicides in the environment usually leads cancer cases that has been reported all over the world (Torres *et al*., 2015).

Weeds cause reduction in soil fertility by hindering the conversion of organic matter into inorganic matter such as Nitrate (NO_3^-), Sulphate SO_3^{2-}, Phosphate (PO_4^-), Potassium (K), Sodium (Na), Calcium (Ca), thereby reducing productivity of crop plants in mechanized and traditional farming. If the application of these herbicides are not restrained it may lead to extreme food shortage in the populace (Kellogg *et al.*, 2000). Selective herbicides usually leave the crop plants relatively unharmed. Selective herbicides usually interfere with the growth of the weeds. The continual usage of herbicides by farmers have shown their importance in mechanized farming. However, the farmers do not know that due to continual application to the soils used for farming, leaching ensues thereby polluting the underground waters which are normally used by the human populace for drinking and cooking and hence persists in top soils (Sangeetha *et al*., 2023). The accumulation of herbicides to toxic levels could results in significant reduction in microbial population, plant population as well as continual desertification. . The exposure of living organisms such as nematodes and microorganisms can result in chronic and acute threats to human health. Long term low dose exposure to herbicides can cause immune suppression, hormonal disruption, diminished intelligence, reproductive abnormalities and carcinoma (Sangeetha *et al.,* 2023).

Uncontrolled application of herbicides to soils could lead to significant depletion of nutrient in the soil or a state where the soil nutrients are adsorbed into the soil chemicals thus making it un-available to plants. Xenobiotics usually circulate within the hydrological cycle, hence their cyclic activities could result in the indiscriminate contamination of the environment, which were initially pristine environments (Gasnier *et al.*, 2009;Giesy *et al.*, 2000;MONSANTO, 2005;Van Bruggen *et al.*, 2018). Furthermore accumulation of the herbicides in the soils have been shown to lead to loss of biodiversity in different areas of activity, thereby affecting the performance of native plants not resistant to herbicides (Batista *et al.*, 2018) Recent agricultural activities which depends on the application herbicides in mechanized farming where the dose application limit is strictly followed may be of good help to the farmer because the weeds are killed and the herbicides applied degrade in good time hence the organic matter are quickly converted to inorganic matter. The plants roots uptake this inorganic matter which thereby leads to effective crop production. Hence the rapid production of crops ensues and these makes the crops available to the populace in affordable quantities.

Bacteria and fungi have been shown by various researchers to be able to break down herbicides thereby utilizing them as source of nutrients thanks to their enzymatic activity which are able to adapt to environments that have been polluted with herbicides. Herbicides are the most effective, efficient and economical way to control weeds and their market continues to grow even with the plethora of generic products. As a result of the development of herbicide resistant crops, the use of herbicides is increasing around the world. Nilesh and Thore (2016), reported that there are now strategies that are now being developed to clean up these pollutants in an economical and eco-friendly manner. Several biological techniques involving biodegradation of organic compounds by microorganisms have been developed. These involves the use of microorganisms adapted to these pollutants to degrade the accumulated herbicides. In the case of mechanized farming, the application of fertilizers to the polluted soils helps in the rapid multiplication of these indigenous microbes leading therefore to acceleration in the rate at which they degrade these pollutants. There is however limited information on microbial bioremediation of herbicide polluted soils among our local farmers who tend to apply herbicides incessantly. Hence this study focused on the

abilities of bacteria and fungi to degrade herbicides (glyphosate, atrazine, paraquat and Xtravest) in polluted soils.

2.0 Materials and Method

Soil Sampling, Soil Treatment and Herbicide Types

The main campus of Olabisi Onabanjo University (OOU) in Ago-Iwoye, Ogun State, Nigeria, is located at 6°55′21″N 3°52′17″E (6.9225, 3.8714). Soil samples were obtained from Olabisi Onabanjo University research plots Ago-Iwoye Nigeria. Several tests (mostly using gas chromatography method) were conducted to determine if the soil samples had been previously polluted with herbicides, the soil samples picked to be used in this work were obtained from a virgin land. The experimental site (open field) was divided into smaller (blocks) of $5m^2$. These treatments include: Control (without treatment) and four individual herbicide (Atrazine, Glyphosate, Xtravest and Gramaxone) treatment plots. The Top soil up to 5 cm depth were collected from herbicide treated soils and control soil samples. Soil augur was used to collect soil samples. The soil samples were sieved through a 2.0mm mesh size to remove stones and plant debris. The herbicides used in this work were obtained from a local agricultural dealership store in Ibadan. The herbicides that were used are; Xtravest, Glyphosate, Gramoxone (Paraquat) and Atrazine.

Soils were treated with the aforementioned herbicides in complete randomized block design for a period of 8 weeks; at company recommended rates of 4 l/h (at 350 ml in 15 l sprayer), soil treatments were carried out in triplicates. (NOTE: No crop plant was present during treatment. Only weeds were present).

Herbicides

The herbicides used in this work were obtained from a local agricultural dealership store in Ibadan. They were; Xtravest (a product of Zhejiang Province Changxing First Chemical Co., Ltd., Xiaopu, Changxing, Zhejiang, China), Glyphosate (Roundup, a product of Monsanto Europe S.A./N.V. Haven 627, Scheldelaan 460 2040 Antwerpen Belgium), Gramoxone (Syngenta Crop Protection AG, Basle, Switzerland) and Atrazine (Forward (Beihai) Hepu Pesticide Co. Ltd., 1, Quingshuijiang Liangzhou Hepu, Beihai, Guangxi, China, 536100**).**

Microbial Isolation, Identification and Determination of Microbial Population

Nutrient agar (NA) was used for the enumeration of total heterotrophic bacteria by the pour plate method. Incubation was done at 30°C for 24-48 h. Potato dextrose agar (PDA) was used for enumeration and isolation of fungi. Incubation of fungal isolates was done at 25°C for 5-7 days. Bacterial and actinomycetes isolates were characterized based on cultural characteristics, staining reactions and biochemical reactions. Identification was thereafter made with reference to Bergey's Manual of Systemic Bacteriology (1984). Starch Casein Agar was used for the enumeration of total actinomycetes count. Morphological and cultural characteristics were used for fungi identification. The bacteria, actinomycetes and fungi that emerged from plates were sub cultured repeatedly until pure cultures were obtained.

Counts of microorganisms were taken from plates containing 15-150 colonies per plates using the Quebec colony counter and this was used to calculate the number of colony forming units per gram (cfu/g) in each soil sample.

Determination of the Biodegradative Abilities of Bacteria and Fungi

The ability of microbial isolates (bacterial consortium, fungal consortium, identified bacteria and identified fungi) to utilise herbicide substrates (atrazine, xtravest, glyphosate and gramoxone) in pure cultures were determined in minimal salt medium (g/l) (Moneke *et al*., 2010). The components were dissolved in 1000ml distilled water, homogenized on hot plate magnetic stirrer to form uniform solution for 30 min. The pH of the basal medium was adjusted to pH 7.2. The basal medium of 150 ml was dispensed into 250 ml Erlenmeyer flasks and herbicide substrates were introduced into each flask respectively at 100 ppm after sterilization which was done separately in an autoclave at 121°C for 15 min and cooled to ambient

temperature. One ml aliquot of diluted overnight broth cultures of each test organisms ($\times 10^4$ cells/ml) were seeded into each flask respectively and the flasks were incubated in a gyratory shaker incubator at 150 rpm for a period of thirty days at 30°C (Bacterial isolates used in this study were those that had the highest turbidity, while the fungal isolates used in this study were those that had the highest counts on each of the herbicides). Utilization of herbicide fractions by microbial isolates was evaluated by monitoring bacterial and fungal growth measured by viable count on nutrient agar. The optical density was determined at 600 nm wavelength with PG T70 U.V/VIS spectrophotometer, changes in pH was determined with pH meter (Model Hama microprocessor P211 pH meter) and fungal dry weights were also determined using the Mettler balance.

Determination of in-vitro Biodegradation of Herbicides by Bacteria and Fungi

The ability of herbicide degrading bacteria and fungi to degrade pure herbicide substrates was tested in minimal salt medium in (g/l) (Na_2HPO_4 2.13g, KH_2PO_4 1.3g, NH_4Cl .5g, $MgSO_4$ $7H_2O$ 0.42g, NaCl} 10g, $NaNO_3$ 0.42g and trace element 1.0 ml) (Moneke *et al*., 2010). Atrazine, xtravest, glyphosate and gramoxone were introduced separately into 100ml Erlenmeyer flasks, at 1.0v/v (concentration 100 ppm), containing 20ml of the basal medium. The flasks were then autoclaved and inoculated with 1.0 ml portion of each isolate (except one which serves as control). The flasks were subsequently incubated in an orbital shaker incubator at 150 rev/min at 30°C for 7days.

Gas chromatography analysis was then used to determine the remaining herbicide after 7 days of incubation in the orbital shaker incubator. The modified method as described by Ayanthi *et al.* (2008) was used in sample preparation and extraction. Extraction was carried out using 100 ml of the sample by adding acetone to sample bound water so that agglomeration of samples during extraction could be minimized, toluene was then added in equal volume of acetone and the samples were agitated using mechanical shaker. The samples were then filtered using vacuum filtration. Acetone was driven off leaving toluene in the mixture.

Organic phase was drained using anhydrous sodium sulphate to remove the associated water during extraction. Methanol was added to dissolve the remaining analytes in the solid phase extracted. The filtrate of the methanol and toluene phase was added together and then allowed to concentrate to about 2.0 ml. The extract was loaded to pre-conditioned C-18 Mega Bond Elute Cartridge. The analytes were eluted from the cartridge with ethyl acetate. The extract was collected and then concentrated with nitrogen stream to about 2.0 ml before gas chromatography analysis was used for the analysis. One micro-liter was injected into the Hewlett Packard Gas Chromatography (HP 6890 Powered with HP ChemStation Rev. A 09.01 [1206] Software).

Mineral Analysis

To 2.0 g of soil sample, 20ml of 1N NH OAC (ammonium acetate solution) was added and the flasks were shaken on a mechanical shaker for 2 h. The mixture was centrifuged at 2000 rpm for 10 min and the clear supernatant was decanted into 100 ml volumetric flasks. About 20 ml of ammonium acetate solution (NH_4OAC) was added twice into the flasks, and shaken on a mechanical shaker for 30 min each, and centrifuged at 2000 rpm and the clear supernatant was then transferred into the same volumetric flasks respectively. Sample extract was made up to 100 ml volume with the NH_4OAC solution.

Sodium, potassium and calcium in the samples were determined using the flame photometer while magnesium in samples was determined using the atomic absorption spectrophotometer fitted with a hollow cathode lamp and a fuel rich flame (air acetylene). Sample solutions (extract) and standard solution for each mineral were injected into the atomic absorption spectrophotometer into sample tray and the mean signal response was recorded for each of the element at their respective wavelength. The concentration of the minerals was calculated (Blakemore *et al*. 1987; USDA, SCS, 1972, 1982).

Soil electrical Conductivity Determination

Electrical conductivity was determined on 50 g of soil mixed with 50 ml of deionized water and the shaken at 200 rpm for 2 h, and then filtered through a filter paper (Grade 4 Whatman International Ltd, Maidstone, England). Electrical conductivity was measured on the filtered solution with an ionic probe (Orion 3 star bench-top conductivity meter Thermo Scientific) (Rhodes, 1996).

Soil Moisture Content

Five grams of soil sample (sieved soil) was weighed into a tarred dish and placed on a Sartorious Moisture content Analyzer. The analyzer temperature was set at 105°C, the analyzer was covered and the moisture content was determined (the values were displayed on the analyser screen).

Soil pH Determination

Twenty grams of air-dried soil was weighed into 50 ml beaker, 20 ml of distilled water was then added and allowed to stand for 30 min and occasionally stirred with a glass rod. The electrode of the pH meter was inserted into the partly settled suspension and the pH was measured after the electrode and pH meter was standardized using buffers pH 4.0 and pH 7.0. (A soil: water ratio of 1:1 was used for the determination of soil pH of herbicide treated soils).

Determination of Available Phosphorus in Soil Samples

Five grams of air-dried soil sample (passed through 2 mM sieve) was weighed into centrifuge tube and 20 ml of extracting solution added. The mixture was then shaken for 1\text{ min} on a mechanical shaker and centrifuged at 2000 rpm for 15 minutes. Two milliliters of clear supernatant was then dispensed into 20 ml test tube with a pipette. Five milliliters of distilled water and 2 ml of ammonium molybdate solution was then added. The content was then mixed properly and 1 ml of SnCl $_2$.2 H 2 O dilute solution was then added and mixed again. After 5\text{ minutes} % transmittance was measured on spectronic-20 spectrophotometer at 660\text{ nm} wave length. Standard curve within the range of 0.1mμ gP/ml (ppm P) was then prepared. Optical density of the standard solution was plotted against the \text{ppm P} and the content of the extractable \text{P} in the soil was calculated (Houba *et al*. 1988)

Determination of total nitrogen in soil samples

Five grams of air-dried soil sample (passed through 2 mm sieve) was weighed into centrifuge tube and 20 ml of extracting solution added. The mixture was then shaken for 1min on a mechanical shaker and centrifuged at 2000 rpm for 15 minutes. Two milliliters of clear supernatant was then dispensed into 20 ml test tube with a pipette. Five milliliters of distilled water and 2 ml of ammonium molybdate solution was then added. The content was then mixed properly and 1ml of $SnCl_2.2H_2O$ dilute solution was then added and mixed again. After 5 minutes percentage transmittance was measured on spectronic-20 spectrophotometer at 660 nm wave length. Standard curve within the range of 0.1mμ gP/ml (ppm P) was then prepared. Optical density of the standard solution was plotted against the ppm P and the content of the extractable P in the soil was calculated (Houba *et al*. 1988)

Determination of Organic Matter in Soil

The percentage organic matter was determined by the method described by FAO (1974). Soil samples were collected and grinded to pass through 0.5 mm sieve. One gram of each soil sample was weighed into 250 ml Erlenmeyer flasks and 10 ml of $K_2Cr_2O_7$ solution was dissolved into each flask and swirled gently to disperse soil. Twenty milliliters of concentrated H_2SO_4 was rapidly added using automatic pipette and swirled gently until the soil and reagents were mixed, then the mixture was swirled more vigorously for one minute, the flasks were then rotated and allowed to stand in a sheet of asbestos for about 30 min. One hundred milliliters of distilled water was added to each flask, followed by 3-4 drops of indicator (ferroin) and titrated with 0.5N Ferrous Sulphate solution to the end point, from greenish or dark green to red (maroon colour), in reflected light against a white background. The organic matter was calculated according to the following formula,

Statistical analysis

The data were statistically analysed, with SPSS version 20 software, using a one-way analysis of variance (ANOVA). Means were compared at 5% level of significance using Duncan's multiple range test.

3.0 Results

Presented in Table 1 are the physicochemical properties and microbial counts of the soil samples used before herbicide treatment. The soil samples type is ferric luvisols and the pH tends towards neutral at 6.8. It was noted that the soil contained high amount of Fe (1820 mg/kg). The soil samples contained much more amounts of sand compared to silt and clay.

Table 1: Physicochemical characteristics of soil samples

Soil properties	Values
Soil type	Ferric luvisols
Bacterial count	9.70
Fungal count (CFU/g×10^5)	1.10
Actinomycetes count (CFU/g×10^5)	0.80
Total nitrogen(%)	0.12
Available phosphorus (ppm)	10.0
Organic matter (%)	2.20
Sodium (cmolkg^{-1})	0.42
Potassium (cmolkg^{-1})	0.44
Calcium (cmolkg^{-1})	4.62
Magnesium (cmolkg^{-1})	1.41
Iron (cmolkg^{-1})	1820
Zinc (cmolkg^{-1})	19
Sulphate (mg/kg)	22.62
Ammonium (mg/kg)	50.30
Nitrate (mg/kg)	41.20
Phosphate (mg/kg)	4910
Soil electrical conductivity (µS/m)	250
pH	6.80
Soil moisture (g)	18.20
Sand (g/kg)	650
Slit (g/kg)	150
Clay (g/kg)	160

In Table 2, treatment of soils with atrazine, glyphosate, xtravest and gramoxone) There were significant differences in the changes observed in bacterial count (P≤0.003), fungal count (P≤0.022) and actinomycetes count (P≤0.004) (Table 2). There were significant differences in the changes observed in bacterial count (P≤0.003), fungal count (P≤0.022) and actinomycetes count (P≤0.004) (Table 2).

In Figure 1, the control recorded higher bacterial counts (4.49×10^3 cfu/g) than the herbicide treated soils. Soils that had been treated with glyphosate had the highest bacterial count of (4×10^3 cfu/g) while xtravest treated soils had the lowest bacterial count of 2.44 ×10^3 cfu/g compared to other herbicide treated soils (Fig. 4).

Table 2: ANOVA table showing the significant level of bacterial, fungal and actinomycetes counts

		Sum of squares	Df	Mean squares	F	Sig.
Soil bacteria count	Between group	7.470	4	1.870	8.270	0.003
	Within groups	2.260	10	0.230		
	Total	9.730	14			
Soil fungal count	Between group	0.030	4	0.230	4.700	0.022
	Within groups	0.010	10	0.010		
	Total	0.040	14			
Soil actinomycetes count	Between group	0.050	4	0.000	7.980	0.004
	Within groups	0.020	10	0.000		
	Total	0.060	14			

In Figure 2 the bacterial population increased from the 2nd to the 6th day of incubation. It was however observed that these indigenous bacterial population started to decline from the 8th to the 14th day of incubation.

Presented in Figs. 2 and 3 are viable counts of bacteria used as biodegradation indices at the 2nd and 4th weeks of herbicide treatment. Viable counts increased from the 2nd to the 6th day of incubation and then dropped from the 8th to the 14th days of incubation. Statistical analysis showed that as the incubation period progressed from the 2nd to the 14th day of incubation. From the 2nd to the 8th weeks of herbicides treatment there were significant changes (P<0.001) in

viable count values. There were significant changes (P<0.001) in viable counts of the herbicide types at the 2nd week of herbicide treatment. Glyphosate had the highest viable count value of 10.520 × 10^5 CFU/g at the 6th day of incubation while gramoxone had the lowest viable count value of 6.100× 10^5 CFU/g at the 14th day of incubation (Fig. 3). In Figs. 4 viable counts increased from the 2nd to the 10th days of incubation and the decreased from the 12th to the 14th days of incubation at the 6th and 8th weeks of herbicide treatment.

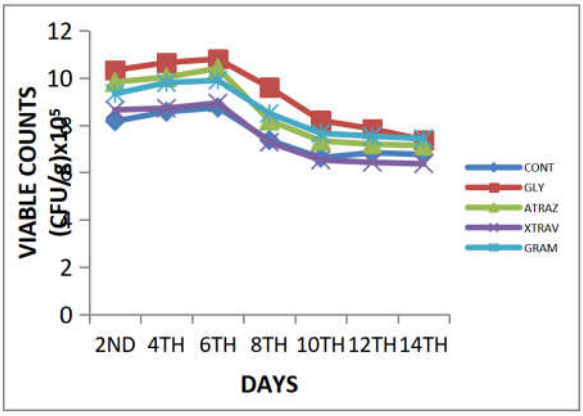

Fig. 1: Changes in viable counts of bacteria during biodegradation at the 2nd week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

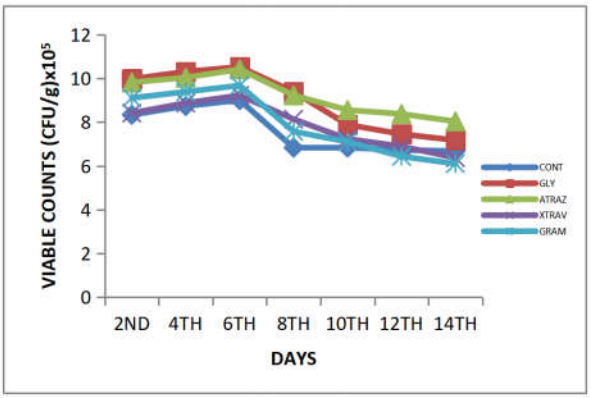

Fig. 2: Changes in viable counts of bacteria during biodegradation at the 4th week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

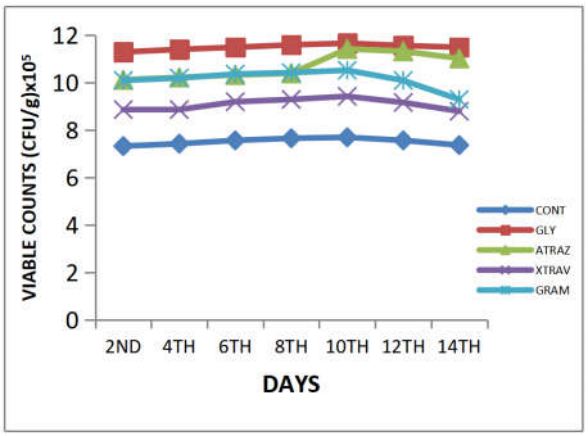

Fig. 3: Changes in viable counts of bacteria during biodegradation at the 6th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and insignificant at P=0.907. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

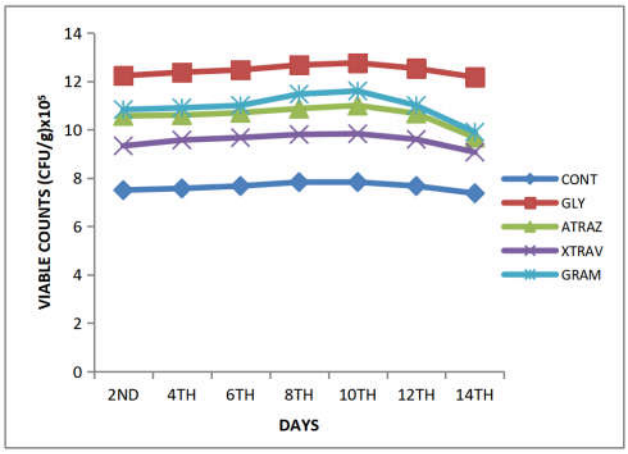

Fig. 4: Changes in viable counts of bacteria during biodegradation at the 8th week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

Presented in Figs.5-8 are changes in optical density of bacteria during biodegradation of herbicides. Weeks of herbicide treatment resulted in insignificant changes (P<0.056) in optical density (Table 7). In Table 8, changes in optical density along the days of incubation and herbicide types were significant (P<0.001). Optical density values increased from the 2nd to

the 14th day of incubation. The highest optical density value of 1.046 was recorded on xtravest at the 14th day of incubation while gramoxone had the lowest optical density value of 0.885 and 0.955 at the 2nd and 14th days of incubation respectively (Fig. 5). In Figs.5-8 all the values of optical density for control were lower than those of the herbicides. Optical density values in gramoxone were found to be lower than those of the other herbicides showing suppressed growth in gramoxone. The highest optical density value of 1.143 was obtained on xtravest at the 14th day of incubation while at the 2nd and 14th days of incubation gramoxone had the lowest optical density values of 0.887 and 0.939 respectively (Fig. 6). In Fig. 7 treatment with each of the herbicide types resulted in significant changes (P<0.001) in optical density values, while along the incubation period optical density values showed high significant (P<0.001) changes. Xtravest had the highest optical density value of 1.465 at the 14th day of incubation while gramoxone had the lowest optical density values of 0.934 and 1.33 at the 2nd and 14th days of incubation respectively.

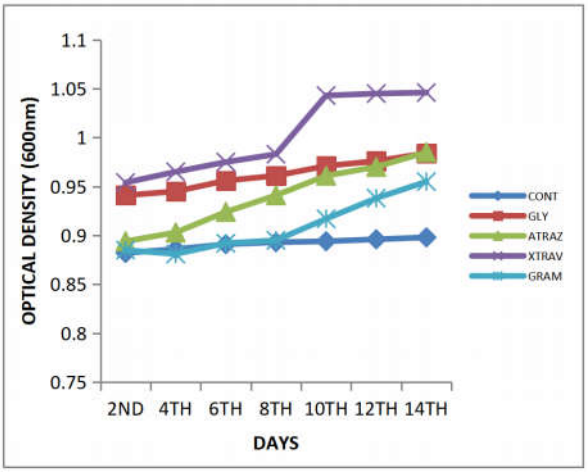

Fig. 5: Changes in optical density during biodegradation at the 2nd week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

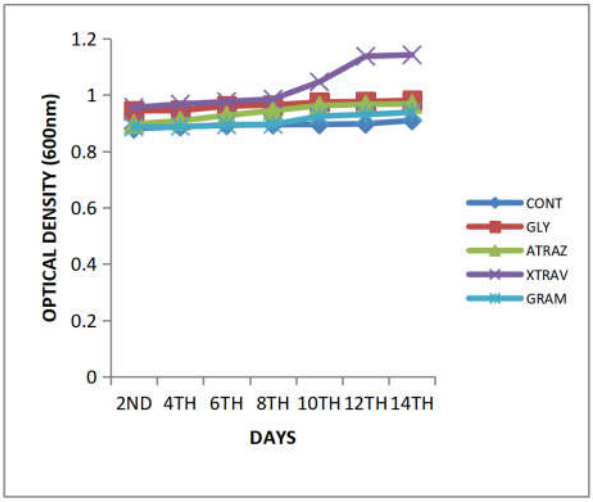

Fig. 6: Changes in optical density during biodegradation at the 4th week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest

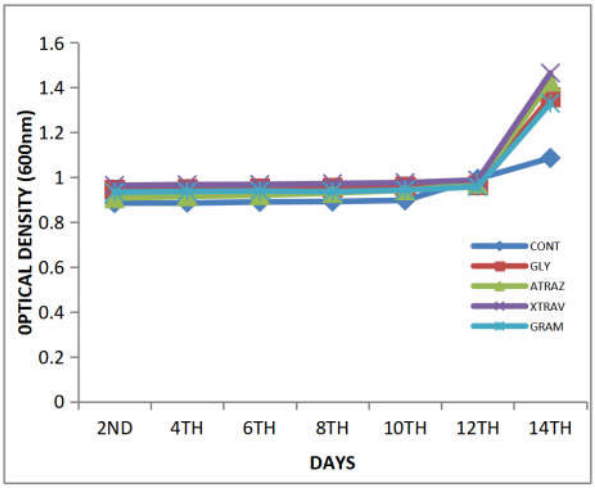

Fig. 7: Changes in optical density during biodegradation at the 6th week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

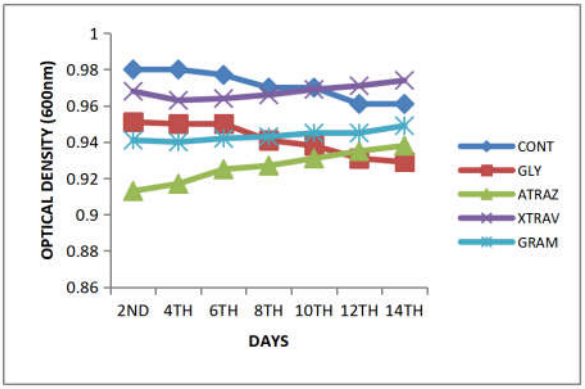

Fig. 8: Changes in optical density during biodegradation at the 8th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and insignificant at P=0.999. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

Presented in Figs. 9-12 are changes in fungal counts during biodegradation from 2nd to 8th week of herbicide treatment. Medium containing glyphosate had the highest fungal count value of 1.37× 10^5 CFU/g at the 20th day of incubation while xtravest had the lowest fungal count value of 0.33× 10^5 CFU/g at the 5th day of incubation, while at the 25th day of incubation of all values recorded xtravest had the lowest fungal count value of 0.77× 10^5 CFU/g. At the 4th week of herbicide treatment fungal counts obtained showed significant changes (P<0.001) in values of fungal counts as produced by each of the herbicide types. Similarly fungal counts showed significant changes (P<0.001) along the incubation period. Fungal counts increased from the 5th to the 15th day of incubation and then decreased from the 20th to the 25th day of incubation. Xtravest recorded the highest fungal count value of 0.8× 10^5 CFU/g at the 15th day of incubation, while gramoxone had the lowest fungal count value of 0.3× 10^5 CFU/g at the 5th day of incubation. At the 25th day of incubation glyphosate had the lowest fungal count value of 0.23× 10^5 CFU/g (Fig.9). In Fig. 10, fungal counts showed significant difference (P<0.001) in values as days of incubation increased from the 5th to the 25th day, while treatment with each of the herbicide types also showed significant changes (P<0.002) in fungal count values. Similar to Fig. 29, fungal count values increased from the 5th to the 15th day of incubation and then decreased from the 20th to the 25th day of incubation. The highest fungal count value of 0.79× 10^5 CFU/g was obtained from xtravest treated soils at the 15th day of incubation while gramoxone recorded the lowest fungal count value of 0.3× 10^5 CFU/g at the 5th day of incubation, while at the 25th day of incubation atrazine had the lowest fungal count value of 0.17× 10^5 CFU/g.

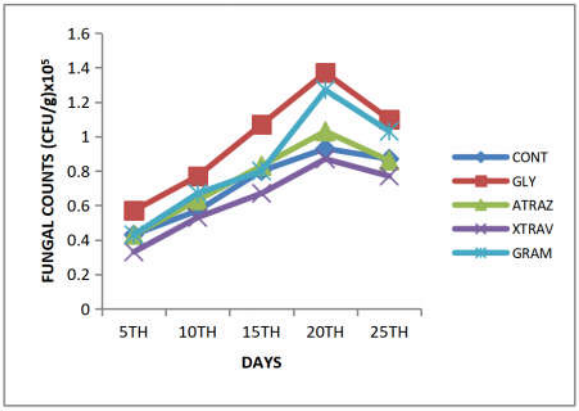

Fig. 9: Changes in fungal counts during biodegradation at the 2nd week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

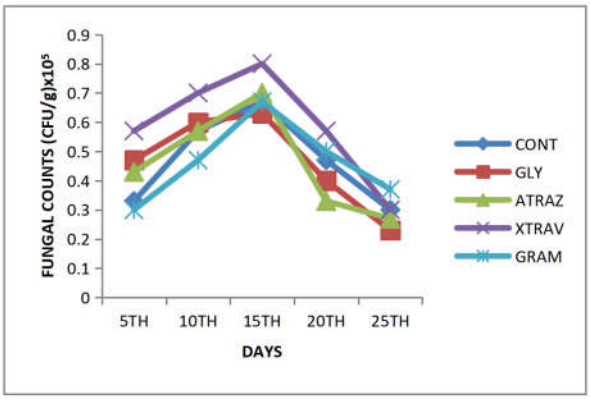

Fig. 10: Changes in fungal counts during biodegradation at the 4th week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

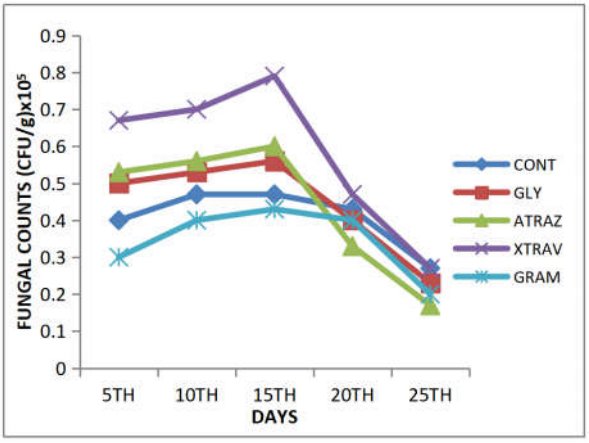

Fig. 11: Changes in fungal counts during biodegradation at the 6th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and P<0.002. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

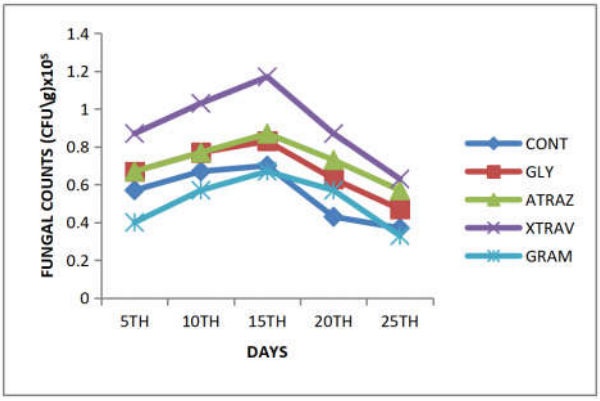

Fig. 12: Changes in fungal counts during biodegradation at the 8th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and P<0.007. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest

Figs. 13-16 show changes in pH during biodegradation of herbicides. Treatment with the herbicide types also caused significant (P<0.001) changes in pH values. Xtravest recorded the lowest pH value of 4.63 at the 14th day of incubation while glyphosate had the highest pH value of 6.51 at the 2nd day of incubation. Xtravest also recorded the lowest pH value of 6.17 of all pH values obtained at the 2nd day of incubation.

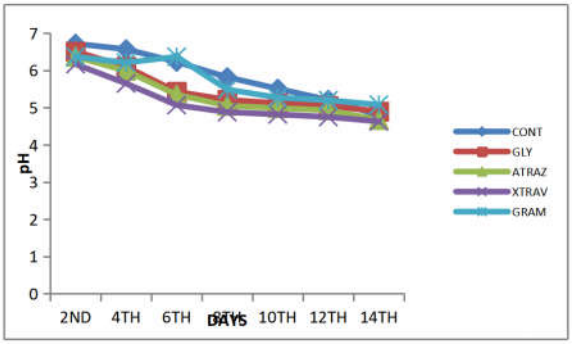

Fig. 13: Changes in pH values during biodegradation at the 2nd week of herbicide treatment. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

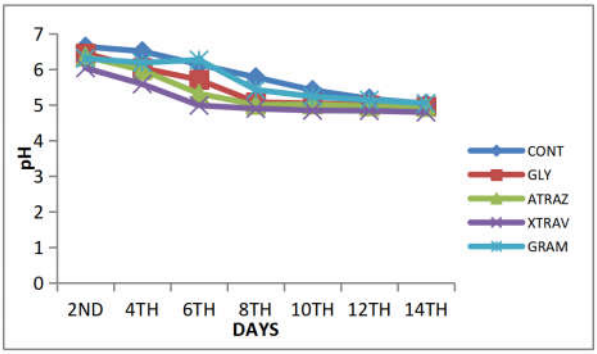

Fig. 14: Changes in pH values during biodegradation at the 4th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and P<0.002. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

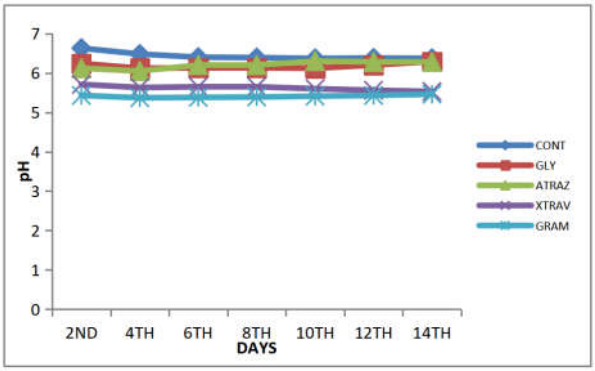

Fig. 15: Changes in pH values during biodegradation at the 6th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and insignificant at P=0.996. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

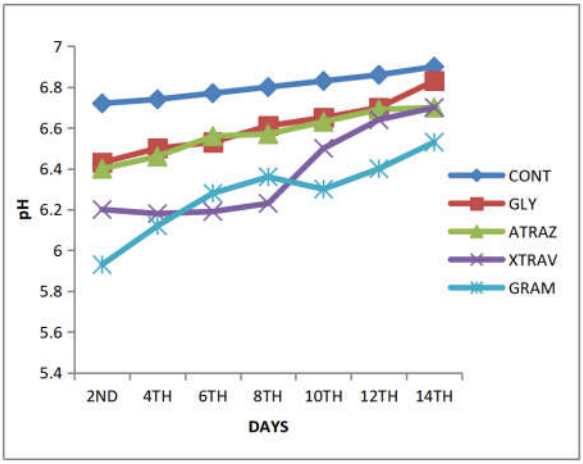

Fig. 16: Changes in pH values during biodegradation at the 8th week of herbicide treatment. ANOVA values are significantly different at P<0.001 and P<0.003. CONT=Control, GLY= Glyphosate, ATRZ= Atrazine, GRAM= Gramoxone, Xtrav= Xtravest.

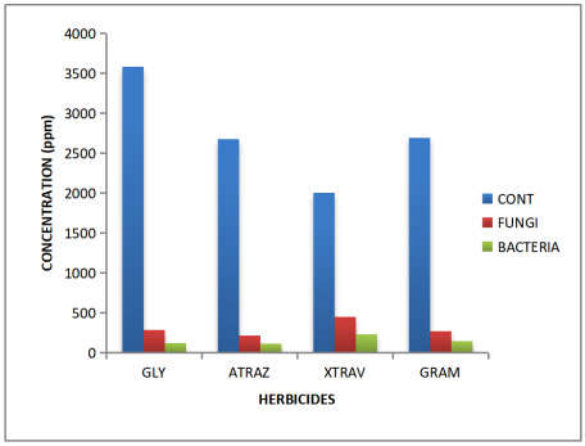

Fig. 17: Biodegradation of herbicides by fungi and bacteria. ANOVA values are significantly different at P<0.001. CONT=Control, GLY= Glyphosate, ATRAZ= Atrazine, GRAM= Gramoxone, XTRAV= Xtravest

In Fig. 17, the concentrations of the control were higher than the concentrations of the bacteria and fungi inoculated herbicides. There were significant changes (P<0.001) in values of the herbicide concentrations after degradation by bacteria and fungi. All herbicides inoculated with bacteria were found to contain lower concentrations of the corresponding herbicide compared to those that had been inoculated with fungi after 7 days of incubation on rotatory shaker incubator. The bacteria and fungi inoculated into each of the herbicides caused sharp reduction in herbicide concentrations compared to the control. The lowest herbicide concentration of 118.55 ppm was obtained from atrazine inoculated with bacteria, while the highest herbicide concentration of 449.23ppm was obtained from xtravest inoculated with fungi at the 7th day of incubation. Atrazine had the lowest herbicide concentration of 220.85 ppm of all herbicides inoculated with fungi, while of all the herbicides inoculated with bacteria xtravest had the highest concentration of 232.79 ppm after 7 days of incubation.

Table 2 shows the bacteria and fungi isolated before and after herbicide treatment. *Pseudomonas* spp were the most prominent bacteria isolated. *P. aeruginosa* occurred much more frequently than all the other bacteria used in this study. Of all the fungi isolated, *A. niger* and *A. terreus* occurred most frequently in culture plates. Indigenous bacteria that adapted to the herbicide treated soils were isolated from the 2nd to the 8th week of herbicide treatment. When the indigenous microorganisms were isolated and identified, *Pseudomonas* spp and *Bacillus* spp occurred frequently in all the herbicide treated soils from the 2nd to the 8th week of treatment. *Actinomyces viscous*, *B. subtilis, P. aeruginosa, P. florescences,* and *P. putida* were also isolated consistently in all the herbicide treated soils. When the fungal population in the herbicide treated soils were analyzed it was shown that *Aspergillus, Fusarium* and *Penicillum* species occurred frequently in all the herbicide treated soils. *A. niger, A. tamarii, F. oxysporum,* and *P. chrysogenum* occurred abundantly in all the herbicide treated soils.

Table 2: Microorganisms isolated from herbicide treated soils

TREATMENT	BACTERIA
Control	*P. aeruginosa, P. florescence, B. cereus, B. subtis, S. aureus, S.faecum A. viscous*
Glyphosate	*B. cereus, B. subtilis, B. macerans, M. luteus P. aeruginosa, P. floresences, P. nigrificans, S. faecum*
Atrazine	*B. macerans, P. aeruginosa, P. florescences, P. putida, S. lactis, Norcadia sp.*
Xtravest	*B. cereus, M. luteus, P. aeruginosa, P. florescences, P. nigrificans, P. putida, S. aureus, A. viscous*
Gramoxone	*B. cereus, B. macerans, P. aeruginosa, P. florescences, P. putida, M. luteus*
Fungi Control	*A.Terreus, A. Tamarii, A. niger, F. oxysporum*
Glyphosate	*A.tamrii, A. niger, A.terreus, F. oxysporum, R. nigricans*
Atrazine	*A.niger, F. oxysporum, A. terrus, A. tamarii*
Xtravest	*A.niger, A. tamarii, A. terreus, Alternaria sp., F. compacticum, P.oxalicum*
Gramoxone	*A.tamarii, A. niger, A. terreus F. oxysporum*

In Fig.18 there were significant differences (P<0.003) in bacterial counts of the herbicide treated soils. The control recorded higher bacterial counts (4.49 × 10^4 cfu/g) than the herbicide treated soils. Soils that had been treated with glyphosate had the highest bacterial count of (4 × 10^3) while xtravest treated soils had the lowest bacterial count of 2.44 × 10^3 cfu/g compared to other herbicide treated soils.

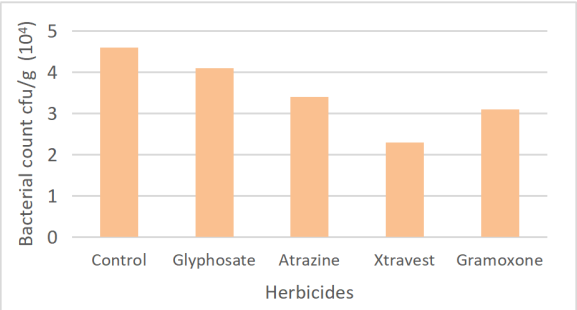

Fig. 18: Bacterial counts of herbicide treated soils

In Fig. 19, Fungal counts of the herbicide treated soils differed significantly (P<0.022). The fungal counts of the control soil samples were higher (3.40 × 10^4 cfu/g) than soils that had been treated with herbicides (Fig. 5). Xtravest treated soils had the highest fungal count of 3.10 \times 10^4 cfu/g while atrazine treated soils had the lowest fungal count of 2.30 \times 10^4 cfu/g. Statistical analysis showed that fungal counts obtained after cultivation were significantly lower (P<0.001) that the values obtained previously at the 8th week of herbicide treatment for all herbicides used in this study.

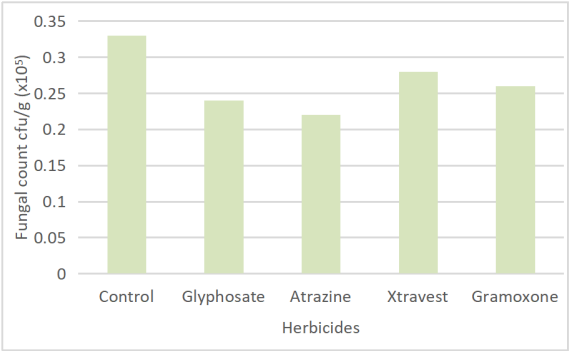

Fig. 19: Fungal counts of herbicide treated soils

Presented in Fig. 20 is the actinomycetes population of herbicide treated soils. There was significant difference (P<0.004) in values of the actinomycetes counts of the herbicide treated soils. The control soil samples recorded higher (4.10 × 10^4 cfu/g) actinomycetes counts than the herbicide treated soils. Of all herbicide treated soils, gramoxone treated soils had the highest actinomycetes count of 4.00 × 10^4 cfu/g while soils that had been treated with xtravest had the lowest actinomycetes counts of 2.60 × 10^4 cfu/g.

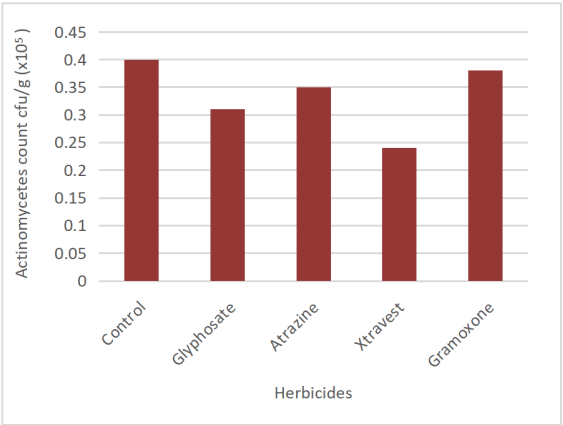

Fig. 20: Actinomycetes counts of herbicide treated soils

Discussion

The bacteria and fungi tested in this study were able to significantly degrade the herbicides. However, bacteria showed better ability to degrade the herbicides because after the biodegradation tests the remaining herbicides were significantly lower than the values obtained for the fungi. The results obtained by Chaussonnerie *et al*. (2015) correlated with what was obtained in this study. Chaussonnerie *et al*. (2015) isolated and characterized two closely related species of *Citrobacter amalonaticus* those were able to transform recalcitrant chlordecone from pesticide contaminated soil. Endosulfan degradation by *P. aeru*ginosa G1 (88.5%), *Stenotrophomonas maltophilia* G2 (85.5%), B. atrophaeus G3 (64.4%), Citrobacter amolonaticus G4 (56.7%) and Acinetobacter lowffii G5 (80.2%) was reported (Ozdal *et al*., 2016).

The proficiency of *Pseudomonas* spp in biodegradation of the herbicides analyzed in this study may have occurred because of the rhamnolipids that are released into the growth medium. The Rhamnolipids emulsifies the substrate hence the substrates turn into oil droplets which increases the surface are of reaction by forming the oil globules. This in turn increases the rate of biodegradation of the herbicides. Bacteria are known to secrete extracellular compounds like lipids which enables rapid biodegradation. The report of Makut and bello (2018) showed that the bacteria isolated were *Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

According to Mat'átková *et al*.(2022), *Pseudomonas aeruginosa* is one of the most studied bacterial producers of biosurfactants. This ubiquitous bacterium can be found in various environments and can also be advantageously used in biotechnological applications, such as microbial fuel cells or desalination cells. These compounds are usually produced as a mixture of congeners, comprised of one or two molecules of rhamnose and one or two molecules of β-hydroxy fatty acid. These can also be accompanied by 3-hydroxyalkanoyloxy-alkanoates (HAAs) which are synthesized as part of the rhamnolipids biosynthetic pathway (Mat'átková *et al*., 2022). The biosynthesis of rhamnolipids involves RhlA, which synthesizes 3-(3-hydroxyalkanoyloxy)alkanoic acids by esterification of two 3-hydroxyacyl molecules bound to acyl carrier protein from the fatty acid de novo synthesis. Subsequently, RhlB catalyzes reaction between an HAA molecule with dTDP-L-rhamnose descending from glucose-6-phosphate to form monorhamnolipids. In the third step, RhlC catalyzes the formation of dirhamnolipids (). Rhamnolipids are known for their excellent surface activities (Mat'átková *et al*., 2022). They can reduce the surface tension of water from 72 mN/m to 30 mN/m (Mat'átková *et al*., 2022) at low critical micelle concentrations. Although rhamnolipids exhibit many activities that predestinate them for use in various industrial applications, the bottleneck of their commercial application is a low production yield and high production cost. For this reason, many studies have been focused on finding a new efficient rhamnolipid producer and/or cost effective bioprocess settings (Mat'átková *et al*., 2022). It is known that the rhamnolipid production is influenced by many factors, including the type of carbon and nitrogen sources, culture conditions and many others. These factors can also influence the rhamnolipid congener composition and therefore the physico-chemical properties of the final product. For rhamnolipid synthesis by *Pseudomonas aeruginosa,* one of the most important factors is the carbon source type and C/N ratio, as the production usually occurs at nitrogen limiting conditions. The optimization of rhamnolipid production can be a time consuming process when individual factors are assessed separately.

These organisms were inoculated on three different herbicides containing mineral salt medium to determine their ability to degrade herbicides. *Bacillus* had the highest percentage of degradation (82.61%) especially in the glyphosate and this is due to the fact that *Bacillus* have competent degradative enzyme, produce spores and they are able the utilize and break the major ingredient in glyphosate which is the (N-(phosphonomethyl)-glycine) and use it as a sole source of phosphorus.

No lag phases were shown in the figures presented in this study showing that the

organisms used in the growth phases had already adapted to the herbicide treatment conditions hence they did not need any lag phase to adapt and grow. They grow straight away after introduction into the culture plates. The bacterial and fungal consortiums were able to utilise the herbicides better than each of the identified fungal isolates. In the work of (Moneke *et al*., 2010)of the seven bacterial species they identified, two (*Acetobacter* sp. and *P. fluorescens*) were selected for further biodegradation studies based on their short lag phase and rapid utilisation of glyphosate. in this study *Pseudomonas* species showed good degradative abilities in cultures containing glyphosate. Matus *et al*. (2023) reported that the studies on the ability of different fungal strains to accumulate and remove pesticides from the environment are mostly focused on (1) indigenous fungi growing at the contaminated sites, or (2) fungi with an enormous physiological capacity to degrade lignin, which is usually associated with the capability to biodegrade xenobiotics. Recently Matus *et al*. (2023) supported the notion that the experimental degradation of organochlorinated herbicide 2,4-dichlorophenoxyacetic acid is usually carried out by bacteria, and therefore, little is actually known about the fungal performance in pesticide's biodegradation

Martins *et al*. (2007) reported that three bacterial strains (*Pseudomonas alcaligenes*, *Enterobacter aerogenes* GC *s.A* and *Klebsiella pneumonia pneumonia* GC *s. B strain 2*.) degraded metolachlor from soils used for mechanised farming. One of them, another strain of *Klebsiella pneumonia pneumonia* GC *s. B strain 2*, isolated from conventional plantation soil was able to metabolize 99% of the herbicide. According to Ferrey *et al*. (1994) metolachlor degradation rate yield was higherthan that of alachlor, another acetanilide compound. The pH values produced by all the bacterial and fungal isolates in culture media showed significant reduction, signifying the production of acidic metabolites. The pH values obtained in fungal culture in this study showed lower pH values than those of the bacterial isolates. The reduction in pH of the culture fluids in the experimental flasks within the incubation periods further confirmed chemical changes of the herbicide substrates which must have been precipitated by microbial enzymes. According to Correa *et al*. (2023), most isolates from genera *Penicillium*, *Aspergillus* and *Trichoderma* probably used the herbicide formulation as a nutrient source. About 8% of the isolates grew better in the medium supplemented with the herbicide than in the conventional culture medium (Czapek). Correa *et al*. (2023) also reported that the isolate *Penicillium* 4A21 produced AMPA and Sarcosine as metabolites from glyphosate degradation. This is the first study of screening for glyphosate degradation carried out with fungi isolated from the Amazon Forest soil. It must be pointed out that extensive use of GP resulted in its accumulation in soil and water environments (Giesy *et al*., 2000; MONSANTO, 2005; Van Bruggen *et al*., 2018). However Van Bruggen *et al*. (2018) reported the negative effects of GP on animal metabolism, in particular, with respect to chronic and remote effects.

According to Matus *et al.* (2023); *Penicillium* sp. was capable of growing with endosulfan, a broad-spectrum cyclodieneorganochlorine insecticide as the sole carbon source. It performed well considering endosulfan biodegradation with almost 95% removal efficiency of its initial 56.7 $mg \cdot L^{-1}$ concentration in six days. However, they carefully referred to this process as the removal or transformation/degradation, since no secondary metabolites have been analysed or shown in the study.

In this study atrazine recorded the lowest concentration of the herbicide after 7 days of incubation while xtravest recorded the highest concentration after 7 days of incubation. The low concentration of atrazine obtained after 7 days of incubation shows that atrazine is the least persistent in soil sample when compared to the other herbicides tested. This however shows that atrazine could be easily biodegraded by microbial consortium resulting in low concentrations after incubation. The highest concentration observed in Xtravest shows its degree of persistence in soils. This might be due to the fact that it is made up of two chemical compounds namely: Atrazine and Metolachlor. Hence its persistence in soils.

Conclusion

This study has shown that the soil indigenous microorganisms could adapt to this chemical stress posed to them and hence biodegrade the herbicides. However, it is noteworthy that to accelerate the rate of biodegradation in herbicide polluted soils it is important to add other growth substrate such as fertilizers. This other substrate helps in the growth and multiplication of this indegenous microbes resulting in faster biodegradation rates. Hence these microorganisms can be used in the bioremediation of pesticide polluted soils.

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