Cumhuriyet Science Journal

e-ISSN: 2587-246X
ISSN: 2587-2680

Cumhuriyet Sci. J., 41(4) (2020) 784-801
http://dx.doi.org/10.17776/csj.774034

The utilisation of herbicides by indigenous microorganisms obtained from Ago-Iwoye, Nigeria, for enhanced growth rates and as carbon source *in-vitro*

Adewole SEBIOMO [1, *], ***Folake M. BANJO*** [1]

[1] *Tai Solarin University of Education, Department of Biological Sciences, Ijagun/ NIGERIA*

Abstract

This study determined the abilities of indigenous microorganisms to utilise atrazine, xtravest, gramoxone and glyphosate as a carbon source and for their growth. Isolation of microorganisms was done using the spread plate method on the solid mineral salts medium with each herbicide added to separate plates. The plates were incubated at 30°C for 5 days for bacteria and at 30°C for 7days for fungi. *B. subtilis, P. aeruginosa, P. florescences, P. putida, Aspergillus niger, A. tamarii, Fusarium oxysporum,* and *P. chrysogenum* were isolated in all the herbicide treated soils. *Bacillus subtilis* recorded the highest optical density value of 1.401 (logCFU/ml) on the 25th day and viable count value of 9.08 (log CFU/ml) (1.21×10^{9} cfu/ml) on the 20th day during growth on glyphosate. *F. oxysporum* recorded the lowest pH of 4 in gramoxone on the 25th day of incubation and the highest count of 6.10×10^{4} cfu/g on the 20th day during atrazine utilisation. *B. subtilis, A. niger* and *F. oxysporum* showed the best abilities to utilise the herbicides for growth and as carbon source. Indigenous microorganisms used in this study successfully utilised the herbicides as carbon source and for growth hence they could be used in bioremediation.

Article info

History:
Received: 26.07.2020
Accepted: 11.11.2020

Keywords:
Herbicides,
Viable Counts,
Biodegradation,
Bioremediation

1. Introduction

Herbicides are used to control weeds in modern agriculture. When herbcides are applied to the field, they control target weeds, as well as have potential residual impact in soil [1]. Varying toxicity of herbicides in soil may cause changes in microbial community structure and function, and concomitantly influencing soil health and ecosystem processes [1]. Microbes are also exposed to these herbicides over a period of time before breakdown by microbes and environmental factors [2].

There are several mechanisms for the clean-up of herbicides in soils, such as chemical treatment, volatilization and incineration [3]. Chemical treatment and volatilization are very effective in herbicide clean up. However, in the long run large volumes of acids and alkalis, which are lethal to the living organisms in the environment are produced and subsequently must be removed. Incineration, which is a very reliable physical-chemical method for destruction of these compounds, has met serious public opposition, because of its potentially toxic emissions, and its elevated economic costs [4]. These physical-chemical cleaning technologies are costly and inefficient because the contaminated soil has to be moved to a storage area where it can be processed [5].

As a result of environmental concerns associated with the accumulation of pesticides in food products and water supplies, it is very important to develop safe, convenient and cheap methods for pesticide removal [4]. Cosequently biological techniques involving biodegradation of organic compounds by microorganisms have been developed [6]. The use of microorganisms, either naturally occurring or introduced, to degrade pollutants is called bioremediation [7]. Fungi are generally more tolerant to high concentrations of polluting chemicals than bacteria [8]. Therefore, some fungi represent a powerful and important tool in soil bioremediation. Some species of fungi have already been used in bioremediation [9]. Glyphosate [N-(phosphonomethyl) glycine, glyphosate], a post-emergence nonselec¬tive broad-spectrum herbicide, and glyphosate-containing herbicides are the most extensively used herbicides in agriculture for the control of many annual and perennial weeds [10-11]. In some cases, glyphosate usage may threaten

*Corresponding author. *Email address: rev20032002@yahoo.com*

agricultural production. Some studies have shown that glyphosate can be broken down by microorganisms and plants. Active glyphosate-degrading microorganisms have been isolated from soils polluted by organophosphonates [12]. Glyphosate is primarily decomposed by bacteria and fungi in the soil. Bacteria and fungi utilize glyphosate as carbon source, leading to the production of ami¬nomethylphosphonic acid, or as a phosphorus source, producing glycine [13]. Bacteria are the most versatile and diversified organisms as a result of their nutritional requirements [14]. İt is important to develop an affordable and environmentally friendly bioremediation method using glyphosate-degrading bacteria as a promising approach for cleans¬ing and restoring soils contaminated with this herbicide [15].

Atrazine is a selective pre- and post- emergence herbicide providing knockdown and residual action. It has low rate of volatilization from soil and is moderately persistent (half life of ~60 days). It is more persistent in neutral and alkaline soils than in soils with low pH [16]. Atrazine is moderately mobile and can be leached through soils into groundwater [17]. Metolachlor is a selective herbicide used in the control of grassy weeds in the cultivation of corn, soybeans, peanuts, cotton and other crops. Metolachlor is often used in combination with other broadleaved herbicides (e.g. atrazine, metobromuron and propazine) to extend the spectrum of activity [18]. Hence this study determined the ability of indigenous microorganisms to utilise atrazine, xtravest (atrazine + metolachlor = xtravest), gramoxone and glyphosate as carbon source and for growth, which is a prerequisite for biodegradation and bioremediation of this herbicides in the soil.

2. Materials and Methods

2.1. Soil sampling

Soil samples were collected from depths of 0–15 cm using soil augur. Samples were then mixed and homogenized. After removing recognizable plant debris, samples were air-dried and sieved through a 2-mm mesh sieve.

2.2. Study site

The present study was carried out in the agricultural field located at Oke Odo Street Ago-Iwoye. The soils had no prior pesticide treatment. The site (open field) was divided into fifteen plots (5 m2 each) of land. The experiment was made up of five treatments (Control (without treatment) and four with treatments).

2.3. Herbicides

The herbicides used in this work were provided from a local agricultural dealership store in Ibadan. They were; Xtravest {atrazine[1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, C8H14CIN5] + metolachlor [2 -chloro -N- (2-ethyl-6-methylphenyl) -N- (1-methoxypropan-2-yl) acetamide], C15H22CINO2, {27+15%} Suspo (SE)} (a product of Zhejiang Province Changxing First Chemical Co., Ltd., Xiaopu, Changxing, Zhejiang, China), Glyphosate [2,4-Dichlorophenoxyacetic acid, C3H8NO5P] (Roundup, a product of Monsanto Europe S.A./N.V. Haven 627, Scheldelaan 460 2040 Antwerpen Belgium), Gramoxone [1,1-Dimethyl-4,4-bipyridinium dichloride, C12H14CI2N2] (Syngenta Crop Protection AG, Basle, Switzerland) and Atrazine [1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine, C8H14CIN5] (Forward (Beihai) Hepu Pesticide Co. Ltd., 1, Quingshuijiang Liangzhou Hepu, Beihai, Guangxi, China, 536100).

2.4. Soil treatments

The treatments 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.

2.5. Isolation of herbicide utilizing bacteria and fungi from soil samples

Five grams of each soil sample was dispensed into 250-ml Erlenmeyer flasks containing a mixture of 50 ml of mineral salts medium and 1 ml of each herbicide in separate flasks. This concentration was used because it is equivalent to the field application rate. The flasks were incubated on a rotary shaker (Gallenkamp, England) at 120 rpm for 7 days at 30°C. Isolation was then carried out using the spread plate method on the solid mineral salts medium with each herbicide added to separate plates. The culture plates were incubated at 30°C for 5 days for bacteria and 30oC for 7days for fungi. The above steps were repeated by taking 1.0 ml of sample from each broth culture and transferred to fresh enrichment medium followed by incubation as described for 7 days. Isolation was done using the spread plate method on the solid mineral salts medium described above with added glyphosate. The plates were incubated at 30°C for 5 days. Morphologically distinct colonies of indigenous microbial isolates of bacteria and fungi isolated on nutrient agar for bacteria and potato dextrose agar for fungi respectively were used in the herbicide utilization experiments. Identity of the bacterial isolates was affirmed after characterization by standard bacteriological methods

[19-20] while the fungal isolates were identified using morphological and cultural characteristics.

2.6. Determination of the abilities of bacteria and fungi to utilise herbicides for growth and as carbon source

The abilities of microbial isolates to utilise herbicide substrates (atrazine, xtravest, glyphosate and gramoxone) in pure cultures were determined in minimal salt medium (g/l) [21]. The components were dissolved in 1000ml distilled water, homogenised 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 sterilisation which was done separately in an autoclave at 121oC for 15 min and cooled to ambient temperature. One ml aliquot of diluted overnight broth cultures of each test organisms (×104 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 30oC (Bacterial isolates used in the utilisation experiments were those that had the highest turbidity, while the fungal isolates used were those that had the highest counts on each of the herbicides). Utilisation 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. A medium without organism was also prepared as control.

2.7. Viable counts

The plates were placed on the Quebec colony counter with the lid removed. Counting was done from the top of the plate using grid lines to prevent counting a colony twice. A mechanical hand counter was used. Every colony was counted regardless of how small or insignificant. The number of the organisms per millilitre of culture was determined by multilying the number of colonies counted by the dilution factor. Viable counts of bacteria were also expressed as log CFU/ml.

2.8. Optical density

A cuvette containing 2 ml of sterile minimal salt medium was inserted into the sample holder of the spectrohotometer (PG T70 U.V/VIS spectrophotometer). The wavelength knob was set at 600nm. The meter was adjusted to read 0% absorbance. The cuvette of the minimal salt medium was removed and the lid closed. The sterile minimal salt medium contained in the cuvette was reinserted to see if the 0% absorbance still registered. Measurements were then made by inserting cuvette containing 2 ml broth culture into the sample holder.

2.9. Dry weights

Filter paper was weighed using the digital mettler balance P 163. The fungi was harvested on the filter aer by filteration and dried in the oven. The weght was then determined. Deductions were then made by sbtracting the weight of the filter aer when it was unused from the weight after harvesting the fungi and oven drying.

2.10. Statistical analysis

The data were statistically analysed, with SPSS 20 software, using a one-way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Microorganisms isolated from herbicide treated soils

Presented in Table 1 are the microbiological and physicochemical properties of the soil at the experimental site before treatment with herbicides. Presented in Tables 2-3 are bacteria isolated from the 2nd to the 8th week of herbicide treatment. Pseudomonas spp and Bacillus spp were found to be of common occurrence in herbicide treated soils from the 2nd to the 8th week of treatment.

B. subtilis, P. aeruginosa, P. florescences and *P. putida* were isolated in all the herbicide treated soils. Of all Actinomycetes species isolated in this study, *Actinomyces viscous* was consistently isolated in all herbicide treated soils. In Table 4 Aspergillus, Fusarium and Penicillum species were of common occurrence in all the herbicide treated soils. *A. niger, A. tamarii, F. oxysporum,* and *P. chrysogenum* were found to be in more abundance in all the herbicide treated soils compared to all other fungal species isolated in this study.

Table 1. Microbiological and physicochemical properties of soil at the experimental site

Soil properties	Values
Soil type	Ferric luvisols
Bacterial count (CFU/ml×10^5)	9.70
Fungal count (CFU/g×10^5)	1.10
Total nitrogen (%)	0.12
Available phosphorus (ppm)	10.0
Organic matter (%)	2.20
Soil electrical conductivity (µS/m)	250
pH	6.80
Soil moisture (g)	18.20
Sand (g/kg)	650
Silt (g/kg)	150
Clay (g/kg)	160

Table 2. Bacterial species isolated at the 2nd and 4th weeks of treatment of herbicides (glyphosate, atrazine, xtravest and gramoxone) on the experimental soil at the rates of 4 l/h (at 350 ml in 15 l sprayer)

TREATMENT	BACTERIA
2nd Week	
Control	*Bacillus cereus, B. subtilis, B. macerans Serratia marscences, Micrococcus acidophilus, M. luteus, Staphylococcus aureus, Pseudomonas nigrificans, P. aeruginosa, P. putida, P. gellucidum, Proteus vulgaricus, Actinomyces viscous, Streptomyces faecalis, Actinomyces sphaerophorum, Norcadia* sp.
Glyphosate	*Aerobacter aerogenes, Bacillus subtilis, B. polymyxa, P. nigrificans, Pseudomonas putida, P. aeruginosa, P. floresences, P. cepaciae, P. gellucidum M. varians, M. luteus, M. acidophilus, Streptococcus lactis, S. aureus, S. marscences, Actinomyces* sp., *Norcadia* sp.
Atrazine	*A. eutrophs, B. licheniformis, B. macerans, B. subtilis, P. purefaciens, P. nigrificans, P. putida, P. stutzeri, S. marscences, M. luteus, Streptococcus zymogenes Actinomyces viscous*, *Norcadia* sp, *Streptomyces ambofaciens*
Xtravest	*A. eutrophs, B. megaterium, B. licheniformis, B. macerans, B. subtilis, Flavobacterium aquantile, P. floresences, P. gellucidum, M. luteus, P. putida, P. nigricans, P. purefaciens, Serratia marscences, Streptococcus faecium, Streptomyces zymogenes, Actinomyces sp., Actinomyces bovis, Actinomyces viscous, Norcadia farcinica*
Gramoxone	*Azotobacter chroccoccum, B. coagulans, B. subtilis, B. macerans, B. cereus, Corynebacterium callunae, M. luteus, M. roseus, P. putida, P. florescences, P. mallei, P. purefaciens, P. nigricans, P. aeruginosa, Serratia marscences Streptococcus faecium, Actinomyces eriksonii, Actinomyces viscous.*
4th Week	
Control	*B. cereus, B. macerans, M. luteus, P. morganii, P. aeruginosa, P. nigrificans, Proteus vulgaricus, Serratia marscences, S. aureus, Streptococcus faecalis, Actinomyces viscous,*
Glyphosate	*Alcaligenes eutrophs, B. subtilis, B. cereus, B. polymyxa, M. luteus, P. aeruginosa, P. gellucidium, P. florescences, P. putida Proteus vulgaricus, S. aureus, Streptococcus lactis, Norcadia sp, Serratia marscences, Actinomyces sp. Streptomyces ambofasciens*
Atrazine	*B. licheniformis, B. subtilis, P. florescences, P. putida, P. purefaciences, P. aeruginosa, P. stutzeri, M. luteus, M. acidophilus, S. aureus, Serratia marscences, Streptococcus zymogenes, Actinomyces viscous*
Xtravest	*A. eutrophs, Bacillus megaterium, P. florescences, P. putida, B. subtilis, B. macerans, B. licheniformis, Flavobacterium aquantile, S. aureus, Actinomyces bovis Norcadia farcinica*
Gramoxone	*Azotobacter chroccoccum, Corynebacterium callunae, B. coagulans, B. macerans, M. luteus, M. roseus, P. aeruginosa, P. chrysogenum, P. florescences P. gellucidum, P. mallei, P. purefaciences, P. putida, Serratia marcences, Streptococcus faecium, Actinomyces viscous, Actinomyces erriksoni*

Table 3. Bacterial species isolated at the 6th and 8th weeks of treatment of herbicides (glyphosate, atrazine, xtravest and gramoxone) on the experimental soil at the rates of 4 l/h (at 350 ml in 15 l sprayer)

TREATMENT	BACTERIA
6th Week	
Control	*B. cereus, B. licheniformis, B. macerans, B. subtilis, M. luteus, P. aeruginosa, P. florescences, P. nigrificans, P. putida, P. stutzeri. Serrratia marcescens, Streptococcus faecum, S. aureus A. Viscous*
Glyphosate	*A. eutrophs, B. macerans, B. polymyxa, Streptococcus faecum, M. luteus, P. aeruginosa, P. cepaciae, P. gellucidium, P. putida, A. viscous, Streptomyces ambofasciens, Norcadia* sp.
Atrazine	*B. licheniformis, B. subtilis, B. cereus, M. acidophilus, M. luteus, P. aeruginosa, P. florescences, P. nigrificans, P. putida, P. stutzeri, S. aureus, Serratia marcences, Actinomyces viscous, Norcadia* sp.
Xtravest	*B. macerans, B. licheniformis, B. megaterium, M. luteus, P. putida, P. florescences, Streptococcus faecum, Serratia marscences, A. viscous, Norcadia* sp.
Gramoxone	*Azotobacter chroccoccum, A. eutrophs, B. coagulans B. macerans, B. subtilis, Corynebacterium callunae, Flavobacterium aquantile, P. mallei, P. putrefaciences, P. aeruginosa, P. gellucidum, P. putida, M. lactis, M. luteus, M. roseus, S. aureus, Actinomyces bovis, Actinomyces erriksonii, A. viscous, Norcadia farcinica*
8th Week	
Control	*B. cereus, B. macerans, B. subtilis, M. luteus, P. aeruginosa, P. florescences, S. faecum, S. aureus, Actinomyces viscous,*
Glyphosate	*A. eutrophs, B. macerans, B. polymyxa, B. subtilis, M. luteus, P. aeruginosa, P. cepaciae, P. gellucidium, P. putida, Streptococcus faecum, A. viscous, Norcadia* sp., *Streptomyces ambofasciens*
Atrazine	*B. licheniformis, B. cereus, B. subtilis, M. luteus, P. aeruginosa, P. florescences, P. gellucidium, P. nigrificans, P. putida, P. stutzeri, S. aureus, Serratia marscences, A. viscous, Norcadia sp.*
Xtravest	*A. eutrophs, B. cereus, B. licheniformis, B. macerans, B. megaterium, B. subtilis, Flavobacterium aquantile, P. florescences, P. putida, P. putrefaciences, Serratia marscences, S. aureus, A. bovis, A. viscous, Norcadia farcinica*
Gramoxone	*Azotobacter chroccoccum, B. coagulans, B. macerans, B. subtilis, Corynebacterium callunae, M. luteus M. roseus, P. gellucidium, P. putida, P. putrefaciences, P. mallei, Proteus morganii, S. aureus, A. Viscous*

Table 4. Fungi isolated after treatment of herbicides (glyphosate, atrazine, xtravest and gramoxone) on the experimental soil at the rates of 4 l/h (at 350 ml in 15 l sprayer)

TREATMENT	FUNGI
2nd Week	
Control	*Aspergillus tamarii, A. niger, F. compacticum, Fusarium oxysporum, Penicillum oxalicum, Alternaria* sp., *Rhizopus nigricans*
Glyphosate	*Alternaria sp., A. terreus, Rhizopus nigricans, A. tamarii, F. compacticum, P. chrysogenum, Penicillum citrinum,*
Atrazine	*A. tamarii, A. terreus, F. oxysporum, P. citrinum, P. oxalicum, R. nigricans, Rhizopus oligosporus*
Xtravest	*A. terreus, A. tamarii, A. niger, P. chrysogenum, F. oxysporum, R. oligosporus, R. nigricans, P. citrinum, F. compacticum, Alternaria* sp.
Gramoxone	*A. tamarii, A. terreus, P. oxalicum, P. citrinum, R. nigricans, F. Oxysporum*
4th Week	
Control	*A. niger, F. oxysporrum, R. nigricans*
Glyphosate	*Alternaria sp., A. niger, A. tamarii, F. oxysporum, R. nigricans, P. oxalicum, P. citrinum, Saccharomyces* sp.,
Atrazine	*A. terreus, A. tamarii, F. oxysporum, R. nigricans, P. chrysogenum, P. oxalicum*
Xtravest	*A. tamarii, A. niger, A. terreus, F. oxysporum, P. chrysogenum, P. oxalicum, R. nigricans, R. Oligosporus*
Gramoxone	*A. niger, A. tamarii, A. terreus, F. oxysporum, P. chrysogenum, P. oxalicum, R. nigricans, Saccharomyces* sp.,
6th Week	
Control	*A. tamarii, A. terreus, A. niger, F. Oxysporum*
Glyphosate	*F. compacticum, P. oxalicum, R. Nigricans*
Atrazine	*A. niger, A. terreus, F. compacticum, P. oxalicum, R. nigricans,*
Xtravest	*A. niger, A. terreus, R. nigricans, P. Oxalicum*
Gramoxone	*A. niger, A. terreus, F. oxysporum, R. nigricans, Saccharomyces* sp.,
8th Week	
Control	*A. niger, A. tamarii, A. terreus, F. oxysporum R. nigrificans,*
Glyphosate	*F. compacticum, P. oxalicum, R. Nigricans*
Atrazine	*A. niger, A. terreus, F. compacticum, F. oxysporum, P. Oxalicum*
Xtravest	*A. niger, A. terreus, F. oxysporum, P. chrysogenum, P. oxalicum, R. Nigricans*
Gramoxone	*A. terreus, F. oxysporum, P. chrysogenum, P. oxalicum, R. nigricans, Saccharomyces* sp.

3.2. Biodegradation indices of identified bacterial and fungal isolates

There were significant changes (P≤0.05) in the optical density and pH values of the identified bacterial isolates on the 5th , 10th, 15th , 20th, 25th and 30th days of incubation respectively during the utilisation of atrazine, glyphosate, gramoxone and xtravest. On the 5th day of incubation during the utilisation of gramoxone the pH values changed significantly (P≤0.05). There was no significant changes (P≥0.05) in viable counts on atrazine on the 30th day of incubation. There were significant changes (P≤0.05) in viable counts of identified bacteria on the 5th, 20th and 25th days of incubation respectively during the utilisation of atrazine. Meanwhile during the utilisation of glyphosate there were significant changes (P≤0.05) in viable counts on the 5th , 25th , and 30th days respectively, while on the 20th day there was no significant change (P≥0.05) in the viable counts of bacterial isolates on the 10th , 15th and 20th days of incubation respectively.

During the utilisation of gramoxone there were significant changes (P≤0.05) on the 5th, 10th, 25th and 30th days of incubation respectively. The changes in viable counts on the 15th and 20th days of incubation were insignificant (P≥0.05). The viable counts during the utilisation of xtravest were found to be significant (P≤0.05) on the 5th, 10th, 15th, 25th and 30th days of incubation respectively. Meanwhile on the 20th day there were insignificant changes (P≥0.05) in the viable counts of the bacterial isolates on the 20th day of incubation.

Meanwhile, *Bacillus subtilis* significantly utilised (P≤0.05) atrazine recording the highest optical density value of 0.934 and viable count value of 8.98 (logCFU/ml) on the 20th day (Figures 1 & 2). Serratia marcescens recorded the lowest viable count and optical density values of 7.78 and 0.549 respectively on the 30th day during growth on atrazine (Figures 1 & 2). *P. putida* had the lowest pH value of 5.84 on atrazine while *P. stutzeri* recorded the highest pH value of 6.42 during growth on atrazine (Figure 3).

Bacillus subtilis recorded the highest optical density value of 1.401 on the 25th day and viable count value of 9.08 (1.21×109 cfu/ml) on the 20th day during growth on glyphosate thus significantly (P≤0.05) utilising glyphosate for growth closely followed by *Pseudomonas aeruginosa* with optical density value of 1.24 also on the 20th day and viable count value of 9.03 (logCFU/ml) (1.07×109 cfu/ml) (Figs. 4 & 5). *Flavobacterium rigense* recorded the lowest viable count and pH values of 7.83(logCFU/ml) and 5.70 respectively on the 30th day during growth on glyphosate (Figures 5 & 6) meanwhile *Streptomyces ambofasciens* had the lowest optical density of 0.614 on the 30th day during growth on glyphosate (Figure 4).

Bacillus coagulans significantly utilised (P≤0.05) gramoxone with peak optical density value of 1.13 and viable count value of 9.14 (logCFU/ml) (1.37×109 cfu/ml) on the 20th day (Figs. 7 & 8). *Micrococcus roseus* had the lowest pH of 5.68 during growth on gramoxone after the 30th day of growth while *Pseudomonas mallei* had the highest pH value of 6.17 after the 30th day of growth on gramoxone (Figure 9). Meanwhile *Corynebacter callaunae* recorded the lowest viable count value of 7.53 (logCFU/ml) (3.4×107 cfu/ml) on the 30th day of growth (Figure 8) while *M. roseus* and *Actinomyces erriksonii* recorded the lowest optical density value of 0.651 after the 30th day during growth on gramoxone (Figure 9).

Bacillus substilis had the highest optical density value of 1.63 and viable count value of 9.25 (logCFU/ml) (Figure 10 & 11) on the 20th day. *Norcadia farcinica* had the lowest pH of 5.97 during growth on xtravest after the 30th day of growth in while *Flavobacterium aquantile* had the highest pH value of 6.34 after the 30th day of growth on xtravest (Figure 12). Meanwhile *A. bovis* recorded the lowest viable count (Figure 11) and optical density values (Figure 12) of 7.83 (logCFU/ml) and 741 on the 30th day of growth on xtravest.

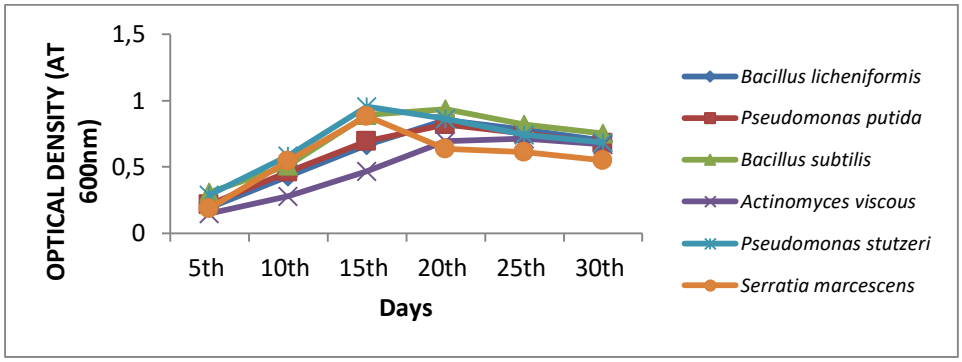

Figure 1. Changes in optical density of bacterial species during growth on atrazine.

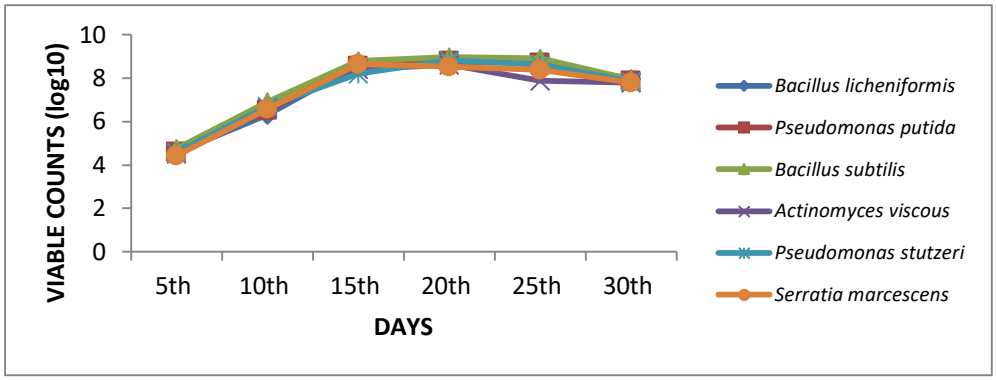

Figure 2. Chages in viable counts of bacteria species, incubated in medium with atrazine herbicide, along the days of incubation were significant for bacteria ($P<0.05$) analysed in this study.

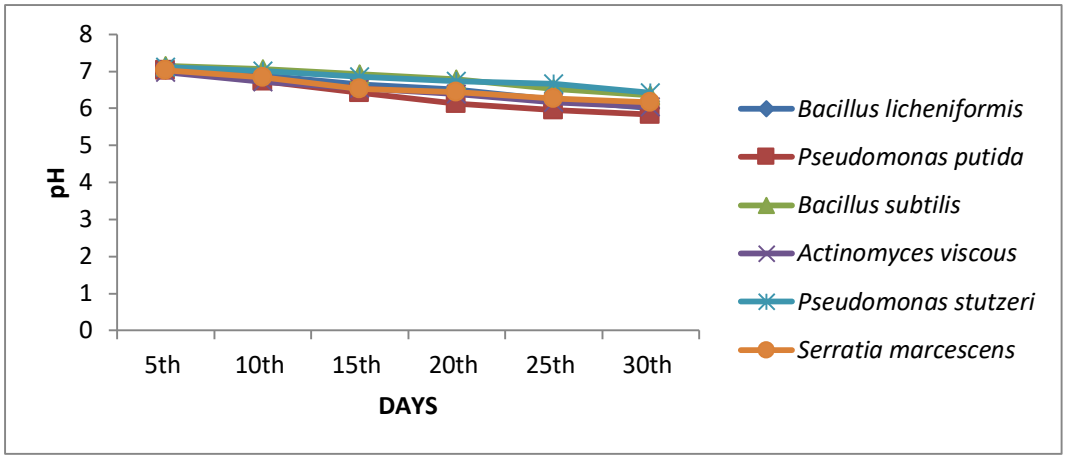

Figure 3. Changes in pH of bacteria species, incubated in medium with atrazine herbicide, along the days of incubation were significant for bacteria ($P\leq0.05$) analysed in this study

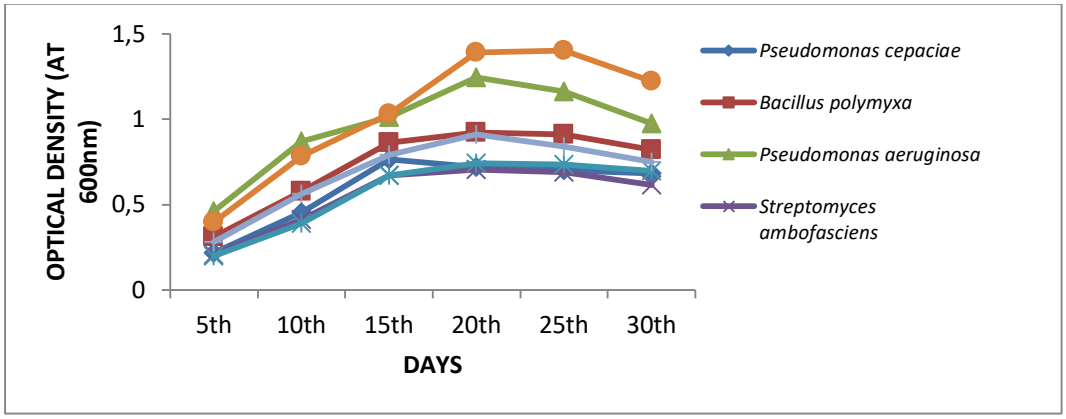

Figure 4. Changes in optical density of identified bacterial species during growth on glyphosate.

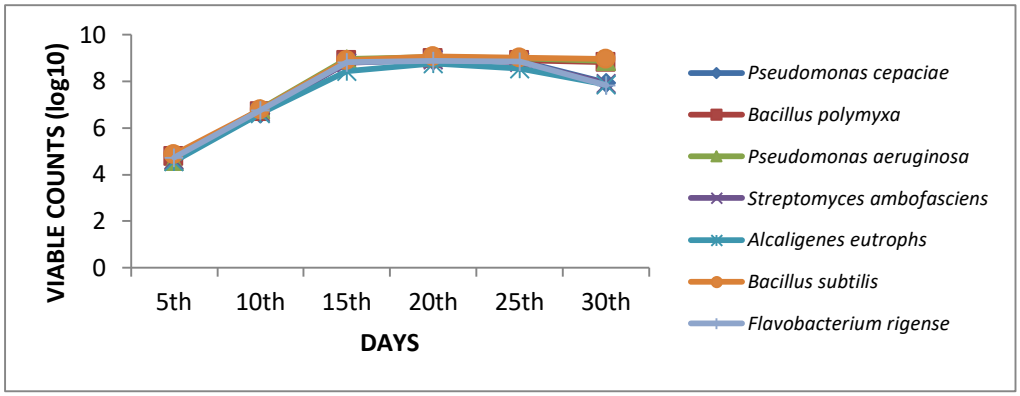

Figure 5. Changes in viable counts of identified bacterial species during growth on glyphosate.

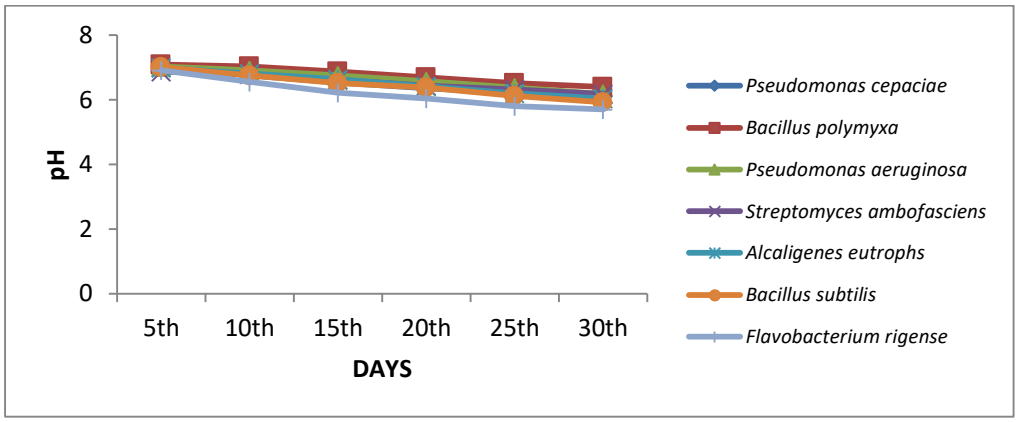

Figure 6. Changes in pH of identified bacterial species during growth on glyphosate.

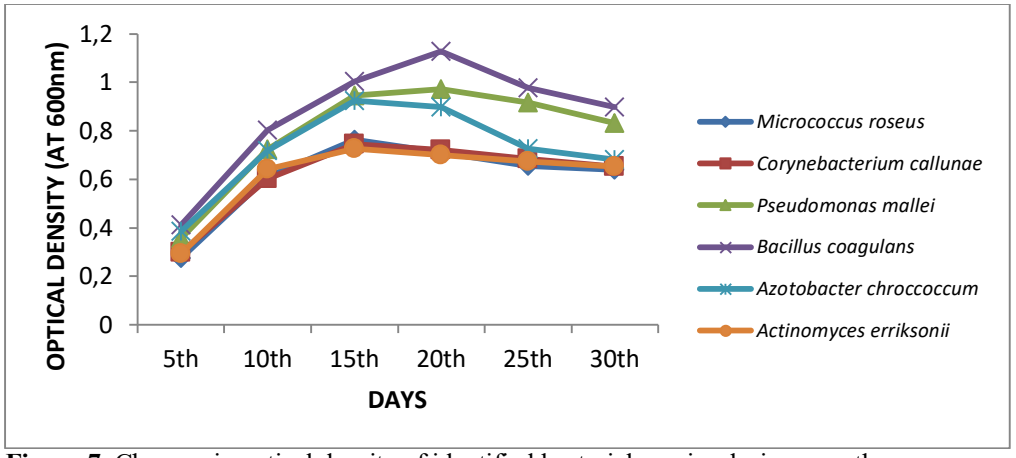

Figure 7. Changes in optical density of identified bacterial species during growth on gramoxone.

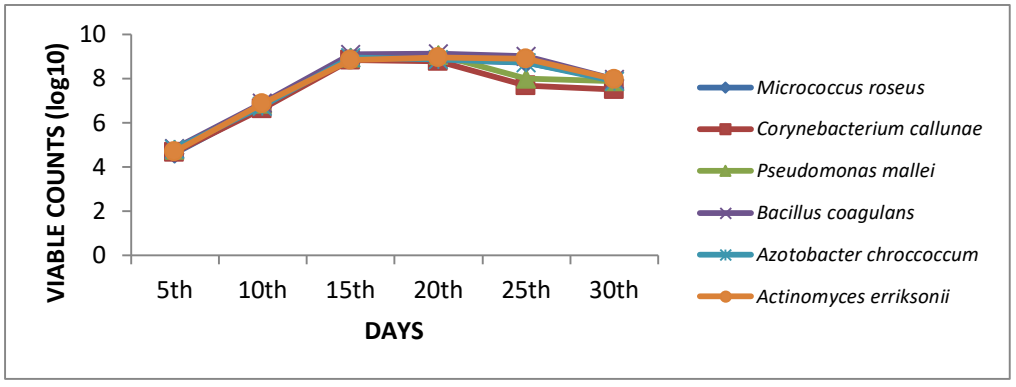

Figure 8. Changes in viable counts of identified bacterial species during growth on gramoxone.

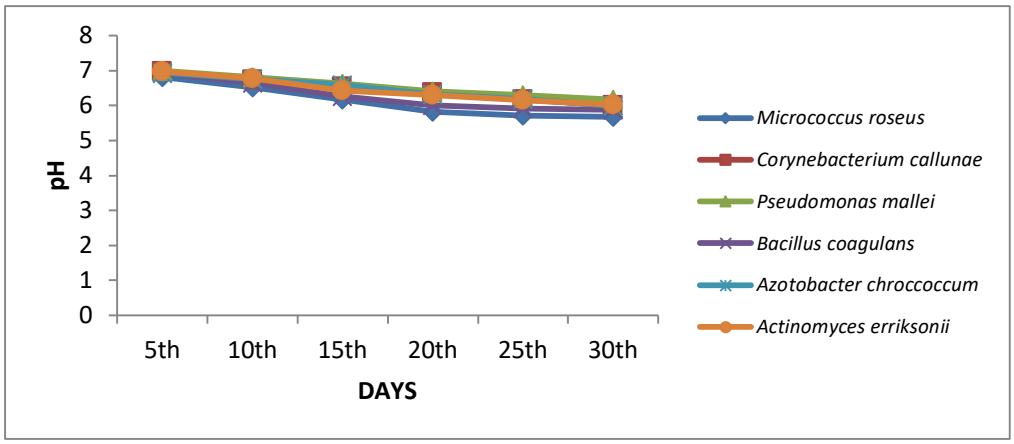

Figure 9. Changes in pH of identified bacterial species during growth on gramoxone.

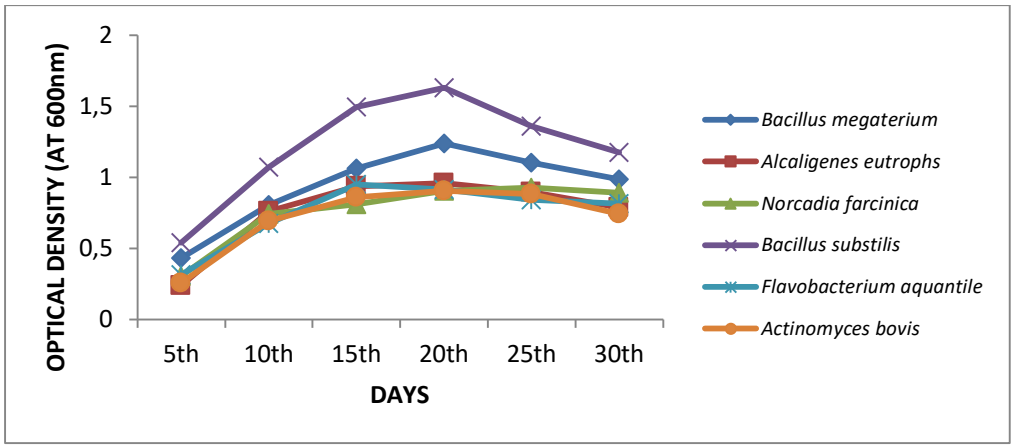

Figure 10. Changes in optical density of identified bacterial species during growth on xtravest.

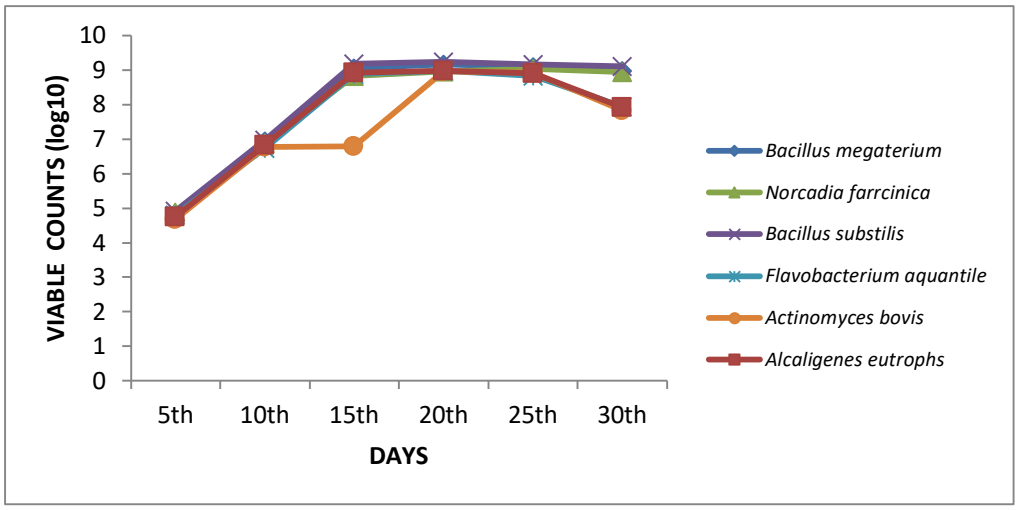

Figure 11. Changes in viable counts of identified bacterial species during growth on xtravest.

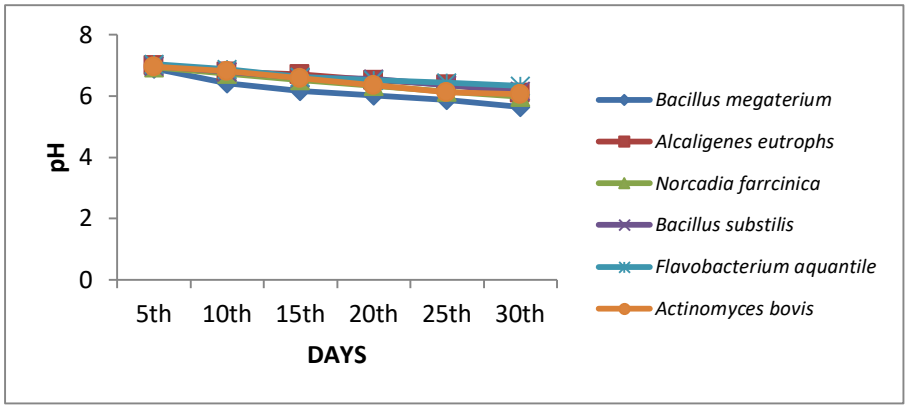

Figure 12. Changes in pH of identified bacterial species during growth on xtravest.

ANOVA results showed that there were significant changes (P≤0.05) in fungal pH values on days 5, 15 and 25 of incubation respectively during utilisation and biodegradation of glyphosate. Meanwhile on days 10 and 20 the fungal isolates showed insignificant difference (P≥0.05) in fungal pH values. In Figures 13 and 16, pH reduced from the 5th to the 25th day of incubation, while in Figures 14 and 15, pH increased slightly from the 5th to the 15th day of incubation and then dropped from the 20th to the 25th day of incubation.

During growth on glyphosate *R. nigricans* and *F. oxysporum* produced the lowest pH value of 5 on the 25th day of incubation; meanwhile A. tamarii had the highest pH of 5.57 and 5.23 on the 5th and 25th days of incubation respectively (Fig. 13).

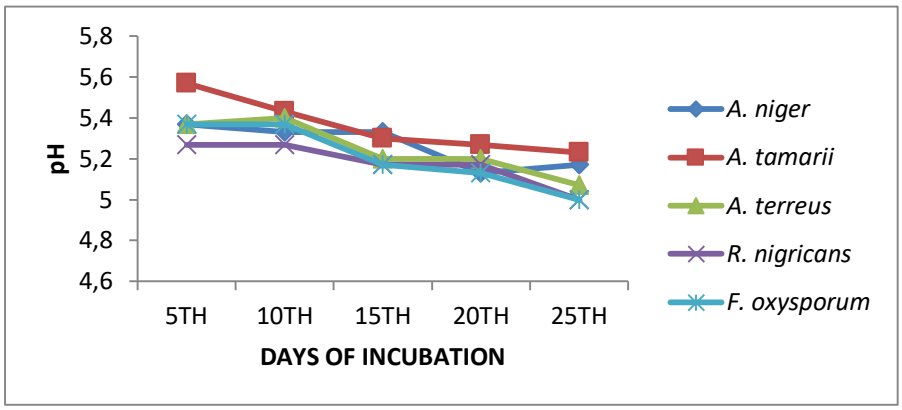

Figure 13. Changes in pH of identified fungal species during growth on glyphosate.

In Figure 14, statistical analysis indicated significant difference (P≤0.05) in pH values of fungi at the 25th day of incubation. There were no significant changes in pH values from the 5th to the 20th days of incubation (P≥0.05). *A. tamarii* had the highest pH value of 5.57 on the 15th day of incubation during growth on atrazine, while *F. oxysporum* had the lowest pH value 5 (Figure 14).

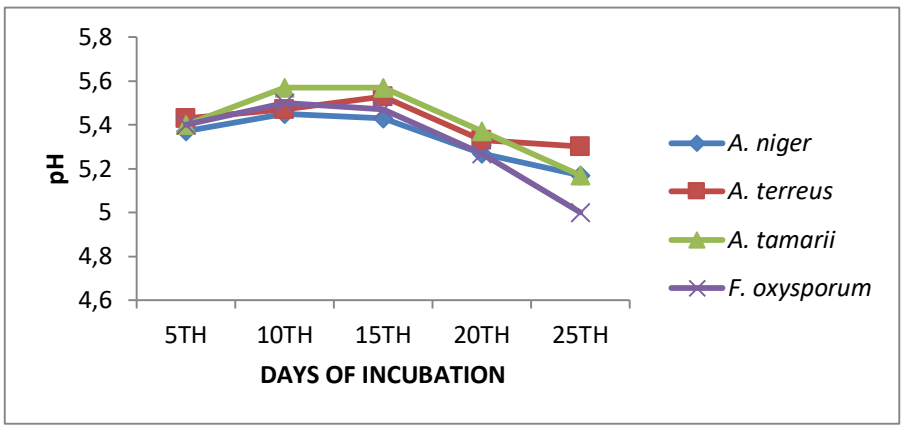

Figure 14. Changes in pH of identified fungal species during growth on atrazine

Statistical analysis showed that there was significant difference (P≤0.05) in pH values of fungal isolates along the incubation period at the 5th, 15th, 20th, and 25th days of incubation respectively. *A. niger* and *P. oxalicum* had the lowest pH value of 5 at the 25th day of incubation during growth on xtravest, while *A. tamarii* had the highest pH of 5.70 on the 15th day of incubation (Figure 15).

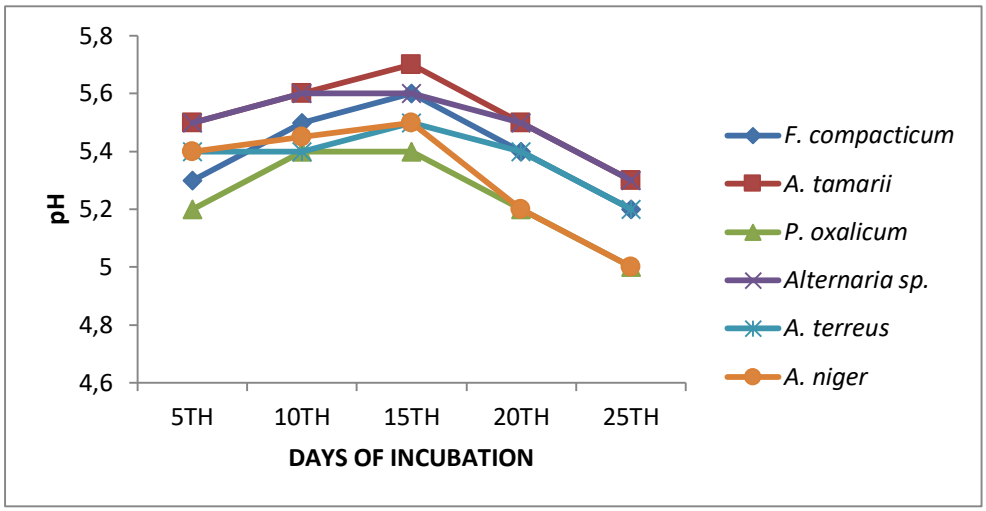

Figure 15. Changes in pH of identified fungal species during growth on xtravest.

In Figure 16, ANOVA results showed that fungi isolates caused significant changes (P≤0.05) in pH values from the 5th to the 25th days of incubation respectively. *F. oxysporum* had the lowest pH values of 4.5 and 4 on the 5th and 25th days of incubation respectively, while *A. terreus* had the highest pH values of 5.6 and 5.3 on the 5th and 25th days of incubation respectively during growth on gramoxone.

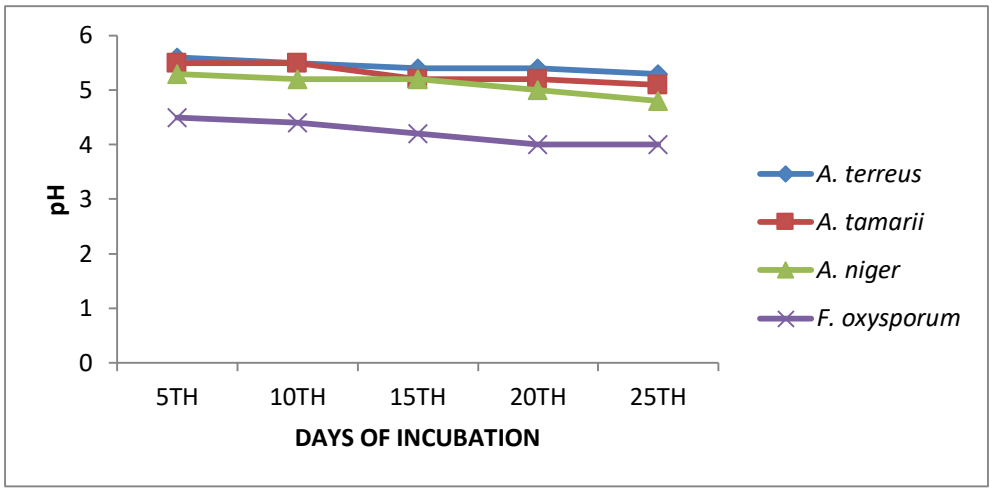

Figure 16. Changes in pH of identified fungal species during growth on gramoxone.

In Figure 17, ANOVA results showed that there was significant difference (P≤0.05) in fungal counts as the incubation period progressed from the 5th to the 25th days of incubation respectively. Fungal counts of *A. terreus*, *R. nigricans* and *F. oxysporum* increased from the 5th to the 20th day of incubation and then dropped on the 25th day of incubation while A. niger increased from the 5th to the 15th day of incubation and then dropped from the 20th to the 25th days of incubation (Figure 17). *A. tamarii* increased from the 5th to the 15th day, remained constant on the 20th and then dropped on the 25th day. *A. niger* had the highest fungal count of 6.00×104 cfu/g on the 15th day of incubation on glyphosate, while R. nigricans recorded the lowest fungal count of 2.00×104 cfu/g on the 10th day of incubation.

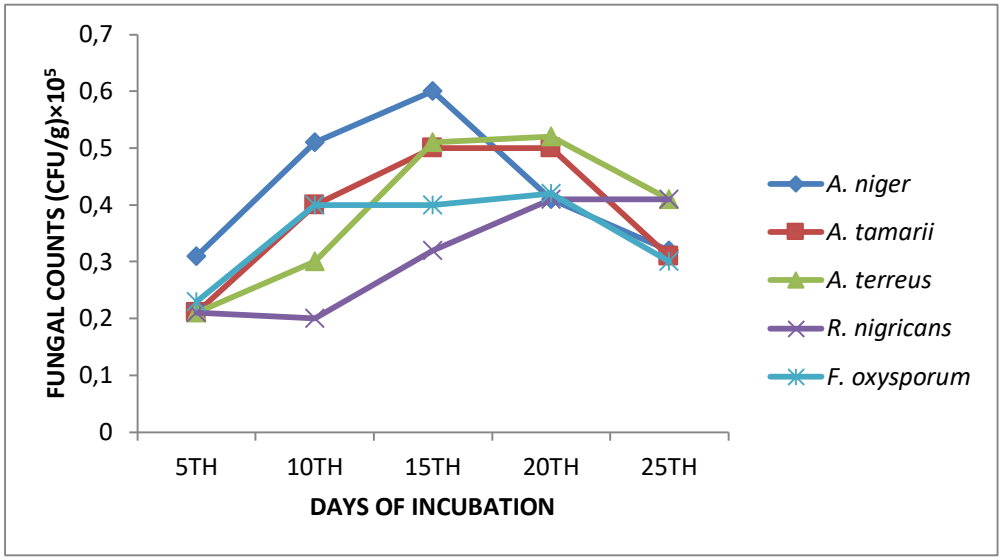

Figure 17. Changes in viable counts of identified fungal species during growth on glyphosate

In Figure 18, statistical analysis indicated significant difference (P≤0.05) in fungal counts along the incubation period from the 5th to the 25th days of incubation respectively. Fungal counts of *A. niger, A. terreus* and *A. tamarii* increased from the 5th to the 15th day of incubation and then dropped from the 20th day to the 25th day of incubation. Conversely counts of *F. oxysporum* increased from the 5th to the 20th day of incubation and then dropped on the 25th day of incubation. *F. oxysporum* recorded the highest count of 6.10×10^4 cfu/g on the 20th day of incubation while *A. terreus* had the lowest count of 2.00×10^4 cfu/g on the 5th day of incubation during the utilisation of atrazine.

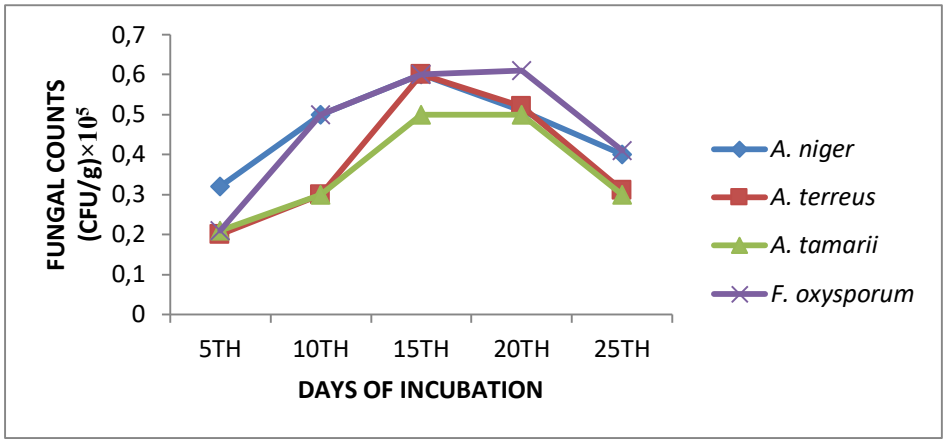

Figure 18. Changes in viable counts of identified fungal species during growth on atrazine

Figure 19 shows changes in fungal counts of identified fungal species during growth on xtravest. ANOVA results showed that there were significant changes ($P \leq 0.05$) in fungal counts among the fungal isolates on xtravest as the incubation period progressed from 5th to the 25th days respectively. Counts of *F. compacticum, Alternaria* sp. and *A. niger* increased from the 5th to the 15th day, remained constant on the 20th day and then dropped on the 25th day of incubation while counts of *A. tamarii, P. oxalicum* and *A. terreus* increased from the 5th to the 20th day and finally dropped on the 25th day of incubation. *A. niger* had the highest count of 6.00×10^4 cfu/g on the 15th and 20th days of incubation, while *P. oxalicum* and *F. compacticum* had the lowest count of 1.10×10^4 cfu/g on the 5th day of incubation.

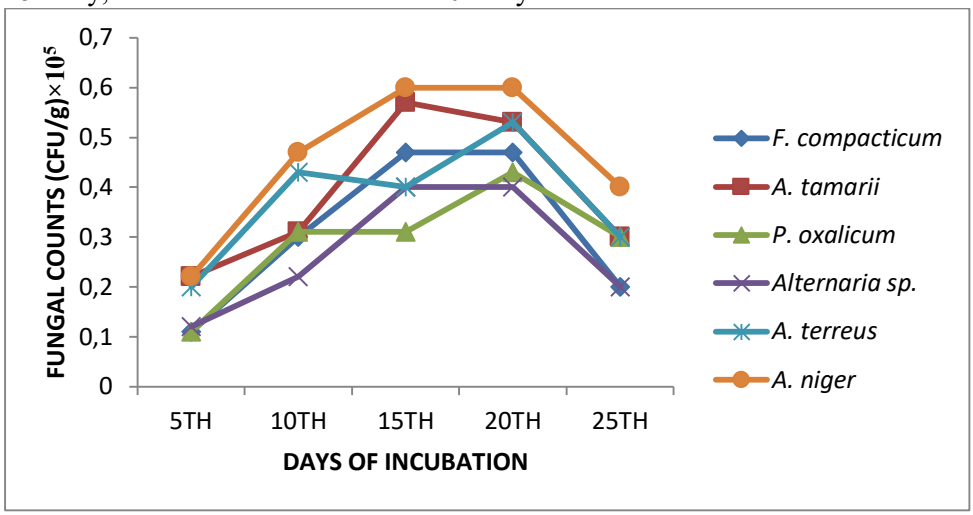

Figure 19. Changes in viable counts of identified fungal species during growth on xtravest. ANOVA values are significantly different at $P<0.001$

Statistical analysis showed that with increase in days of incubation, changes in fungal counts of the fungal isolates on gramoxone were significant ($P<0.009$, $P<0.001$, $P<0.006$, $P<0.10$ and $P<0.002$) from the 5th to the 25th days of incubation respectively (Figure 20). Counts of fungal isolates, *A. terreus, A. tamarii, A. niger* and *F. oxysporum*, increased from the 5th to the 15th day and then dropped from the 20th to the 25th day of incubation. *F. oxysporum* recorded the highest fungal count of 5.00×10^4 cfu/g on the 15th and 20th days of incubation. *F. oxysporum* also recorded the lowest fungal count of 1.00×10^4 cfu/g on the 5th day of incubation (Figure 20).

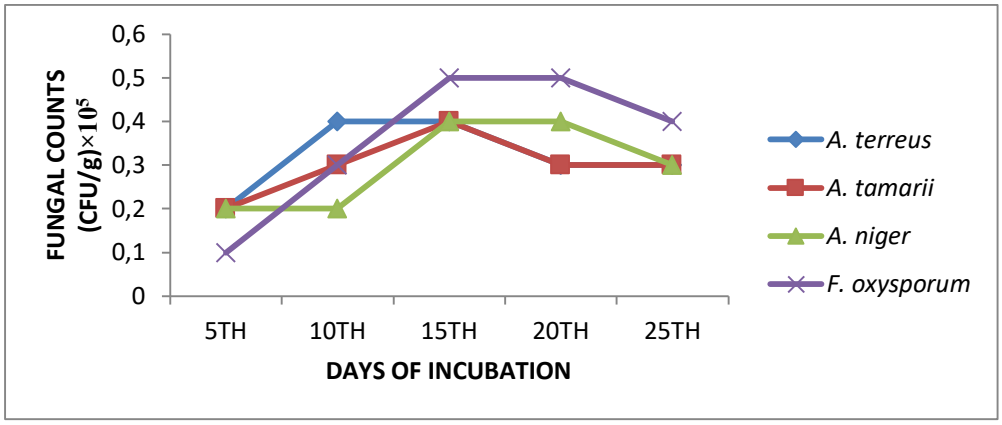

Figure 20. Changes in viable counts of identified fungal species during growth on gramoxone.

Results of analysis of variance showed that there was no significant difference (P≥0.05) in the fungal dry-weight values of each fungal isolate at the 5th, 10th, 20th, and 25th days of incubation respectively. However there were significant differences (P≤0.05) in dry-weight values of the fungal isolates on the 15th day of incubation (Figure 21). During the utilization of herbicides for growth dry-weight values increased from the 5th to the 15th days of incubation, remained constant to the 20th day and the dropped on the 25th day of incubation. *A. tamarii* had the highest dry-weight value of 0.006g at the 15th and 20th days of incubation, during growth on glyphosate (Figure 21), while on the 5th day of incubation *A. terreus* had the lowest dry-weight value of 0.002g.

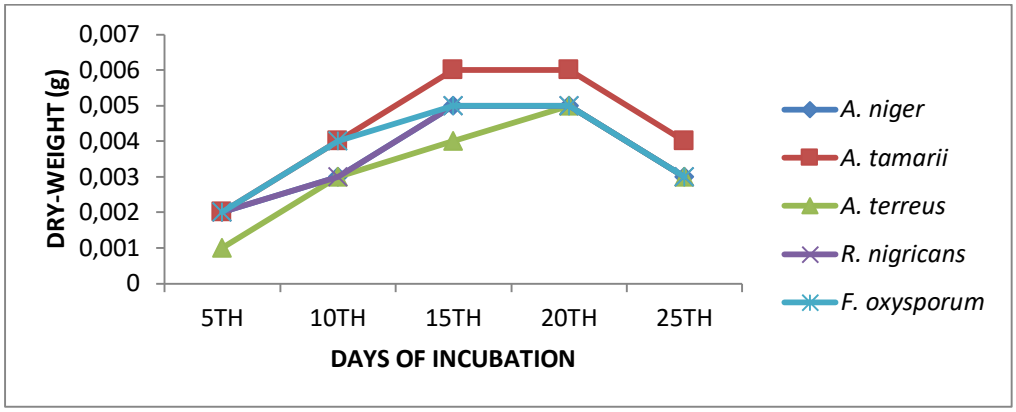

Figure 21. Changes in dry-weights of identified fungal species during growth on glyphosate.

ANOVA results showed that there were insignificant changes (P≥0.05) in dry-weight values of the fungal isolates from the 5th to the 25th days of incubation respectively (Figure 22). Meanwhile on the 25th day of incubation, there was significant change (P≤0.05) in dry-weight values of the fungal isolates. *A. niger* recorded the highest dry-weight value of 0.006g at the 20th day of incubation (Figure 22) during growth on atrazine *A. terreus* had the lowest dry-weight value of 0.002g at the 25th day of incubation on atrazine.

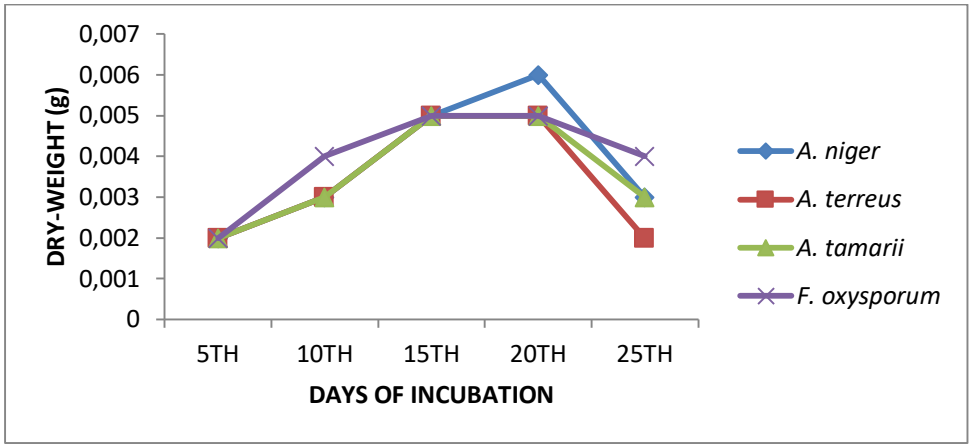

Figure 22. Changes in dry-weights of identified fungal species during growth on atrazine

In Figure 23, ANOVA results showed that there were significant changes (P≤0.05) in dry-weight values from the 5th to the 20th days of incubation respectively while on the 25th day of incubation there was no significant change (P≥0.05) in dryweight values of the fungal isolates. *P. oxalicum* had the highest dry-weight value of 0.007g on the 20th day of incubation on xtravest, while *F. compacticum* and *A. terreus* had the lowest dry-weight value of 0.001g at the 5th day of incubation.

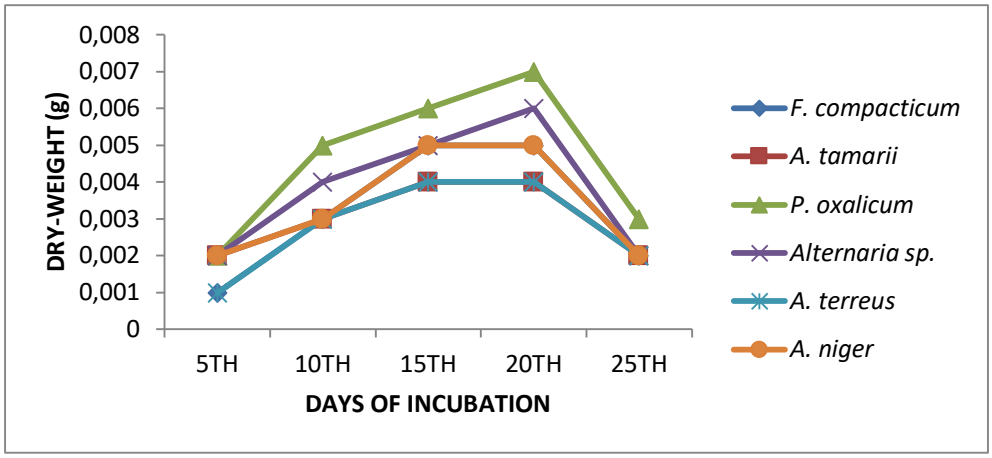

Figure 23. Changes in dry-weights of identified fungal species during growth on xravest.

Statistical analysis showed significant changes ($P \leq 0.05$) from the 5th to the 20th day respectively. On the 25th day there was no significant change ($P \geq 0.05$) in dry-weight values of the fungal isolates (Figure 24). *A. niger* and *F. oxysporum* recorded the highest dry-weight value of 0.006g at the 15th day of incubation during growth in gramoxone, while *A. terreus* and *A. tamarii* had the lowest dry-weight value of 0.002g at the 5th day of incubation.

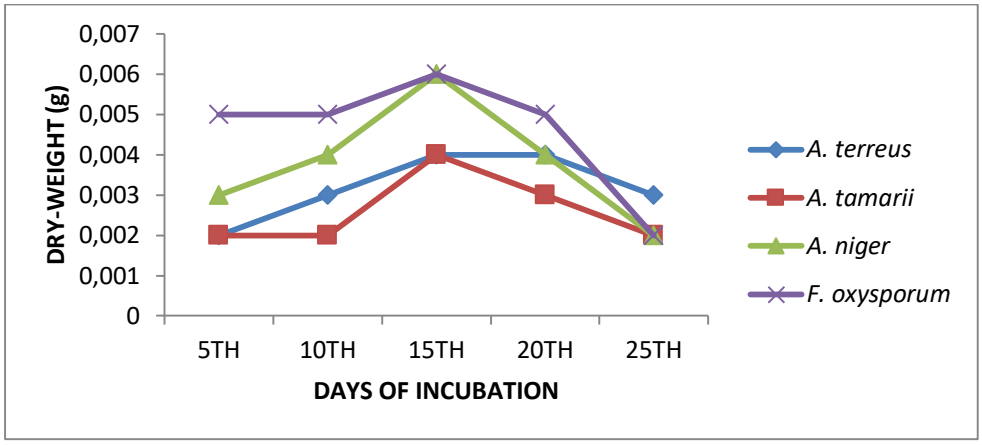

Figure 24. Changes in dry-weights of identified fungal species during growth on gramoxone.

The isolation of bacteria (*P. florescences, P. putida, P. aeruginosa* and *P. fluorescens*, *Bacillus*, *B. subtilis, B. cereus*, *Actinomyces viscous*, *Actinomyces bovis, Actinomyces eriksonii, Actinomyces sphaerophorum, Norcadia* sp., *Norcadia farcinica, Streptomyces ambofaciens* and *Streptomyces faecalis)* and fungi (*A. niger, A. tamarii, F. oxysporum* and *P. chrysogenum*), from the herbicide treated soils, on minimal salt media is an indication of the adaptation of the indigenous microbial flora to the herbicide treated environments, their ability to utilise the herbicides as carbon source and their consequent biodegradation ability. Several works have reported that native microorganisms from soil and sediment are capable of degrading pesticides (22). Chirnside *et al.* (23) studied indigenous microbial consortium isolated from contaminated soils to determine its potential to degrade atrazine and alachlor. The result obtained in this study was also consistent with the report of Moneke *et al.* (21) which stated that twelve bacterial isolates were initially isolated from rice field samples, which had been previously exposed to glyphosate based formulation (Roundup) for long periods of time. The twelve bacteria isolates were able to grow in the presence of glyphosate as sole phosphorus source. On further sub-culturing on solid media enriched with glyphosate, only five showed the capacity to grow in the presence of the herbicide. The five bacterial isolates were identified as *Acetobacter* sp., *Escherichia* sp., *P. fluorescens, Azotobacter* sp., and *Alcaligenes* sp. Whitelaw-Weckert *et al.* (24) isolated *Pseudomonas* spp. from glyphosate, diquat, paraquat and carfentrazone-ethyl treated soils. Similar to what was obtained in this study; Filimon *et al.* (25) reported the prevalence of the following *Streptomyces* species in their herbicide treated soil samples: *Streptomyces*

aureus, Streptomyces albus and *Streptomyces chrysomallus*. Many studies (26; 27; 28) have also shown that bacteria and fungi like: *Arthrobacter*, *Pseudomonas*, *Bacillus*, *Actinomycetes*, *Mycoplana*, *Agrobacterium*, *Corynebacterium*, *Flavobacterium* and *Norcadia* are herbicide degrading microorganisms.

Although bacteria are considered to be most important group in herbicide degradation, also fungal species have been discovered in soil, with important biodegradation role (25). Thus, fungi species plays an important role in glyphosate degradation, *Mucor, Fusarium,* and *Trichoderma* strains seems dominant in degradation of these herbicide, using glyphosate as source of C, N and P (29;30). Ayansina and Oso (18) isolated *A. flavus, A. niger, Penicillum* sp, and *Trichoderma* sp., from atrazine and Atm (atrazine + metolachlor) treated soils.

The bacterial and fungal isolates used in the determination of the time utilisation of the herbicides showed appreciable growth in culture medium containing the herbicides as carbon source. The differences observed in the growth of the isolates in the media are indications of the differences between the organisms in tolerating the herbicides. The observable differences in the lag and stationary phases also indicates the differences in the abilities of the test indigenous microorganisms to biodegrade the herbicides. This study showed that the bacterial and fungal isolates grew maximally on all the herbicides especially *Bacillus subtilis* which completely utilised atrazine and xtravest in culture recording the highest viable counts and optical density. Similar report was obtained by Dibua *et al*. (31); they stated that in their experiment the efficient utilization of the medium by *Bacillus subtilis* was apparent. A very sharp increase in growth rate with correspondingly sharp decline in pH of the medium was observed following 24 h incubation. In spite of the normal microbial growth curve, however, there was no apparent lag phase: a peak in the growth occurred at 72 h of incubation, followed by a long stationary phase, a decline phase after 192 h of incubation at a pH of 3.7.

Meanwhile of all the fungal isolates used in this study *F. oxysporum* and *A. niger* showed the best abilities to utilise the herbicides because they grew maximally on all the herbicides recording the highest fungal counts. According to Adelowo *et al.* (32), the isolated fungi species *(Trichoderma viride, A. niger, and F. oxysporum)* were able to grow in glyphosate enriched medium. *Trichoderma speccy* showed the highest growth population (with maximum absorbance of 2.40), while *A. niger* showed the lowest growth population (with maximum absorbance of 0.60). The rate of growth of *T. viride* in glyphosate enriched medium was compared with culture medium containing no glyphosate through spectrophotometric analysis. It was observed that the rate of growth of fungus was more rapid in glyphosate enriched culture medium than in culture medium containing no glyphosate. The population of all the fungi isolates increased initially. The increasing trend continued within six to seven days and decreased thereafter. All these were pointer to the fact that fungi species could utilize glyphosate for their growth.

The growth profiles have shown that none of the bacterial and fungal isolates exhibited lag phases, because the microorganisms used in this study are indigenous to the soil from which they were obtained and consequently have adapted to the herbicides used in treatment. Bacterial and fungal consortiums were able to utilise the herbicides better than each of the identified bacterial and fungal isolates. In the work of Moneke *et al.* (21), 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. Martins *et al.* (33) reported the ability of *Klebsiella pneumonia pneumonia* GC *s. B strain1*, *Pseudomonas alcaligenes*, *Enterobacter aerogenes* GC *s.A* and *Klebsiella pneumonia pneumonia* GC *s. B strain 2* to utilise metolachlor with *Pseudomonas alcaligenes* demonstrating the fastest bacterial growth rate in a selective medium than in a rich culture medium. Metolachlor degradation rate yield was far superior to that shown by alachlor, another acetanilide compound (34).

The pH values produced by all the bacterial and fungal isolates in culture media reduced significantly, signifying the production of acidic metabolites. The pH values of the fungal isolates used in the herbicide utilisation experiment showed lower pH values than those of the bacterial isolates. The fungi *F. oxysporum* recorded the lowest pH of 4.5 in gramoxone. 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. Dibua *et al.* (31) also reported similar observations.

Conclusion

B. subtilis, F. oxysporum and *A. niger* showed the best abilities to utilise the herbicides for growth and as carbon source. Indigenous microorganisms used in this study successfully utilised the herbicides as carbon

source and for growth. Hence the indigenous microorganisms could be employed in the bioremediation of herbicide polluted soils. The ultimate success of bioremediation is dependent on microorganisms staying in close physical contact with substance to be degraded. The key to increasing the rate of biodegradation of herbicides in the soil is to optimise the growth rate of indigenous soil degrading micro flora.

Conflict of interest

The authors state that there is no conflict of interests.

References

[1] Zabaloy M.C., Garland J.L. and Gómez M.A., An integrated approach to evaluate the impacts of the herbicides glyphosate, 2,4-D and metsulfuron-methyl on soil microbial communities in the Pampas region, Argentina, *Appl. Soil Ecol.*, 40 (2008) 1–12

[2] Pampulha M.E. and Oliveira A., Impact of an herbicide combination of bromoxynil and prosulfuron on soil microorganisms. *Curr. Microbiol.*, 53 (2006) 238–243

[3] Teng Y., Luo Y.M., Sun X.H., Tu C., Xu, L., and Liu W.X. Influence of arbuscular mycorrhiza and *Rhizobium* on phytoremediation by alfalfa of an agricultural soil contaminated with weathered PCBs: A field study, *International Journal of Phytoremediation* 12 (5) (2010) 516-533.

[4] Zhang J. and Chiao C., Novel approaches for remediation of pesticide pollutants, *International Journal Environment and Pollution*, 18 (5) (2002) 423-433.

[5] Kearney P. and Wauchope R., Disposal options based on properties of pesticides in soil and water, in: P. Kearney, T. Roberts (Eds.), Pesticideremediation in Soils and Water, Wiley Series in Agrochemicals and Plant Protection, (1998).

[6] Schoefs O., Perrier M. and Samson R., Estimation of contaminant depletion in unsaturated soils using a reduced-order biodegradation model and carbon dioxide measurement. App., *Microbiol. Biotechnol.*, 64 (2004) 256-261.

[7] Pointing S.B., Feasibility of bioremediation by white-rot fungi. *Appl. Microbiol. Biotechnol.*, 57 (2001) 20-33.

[8] Evans C. and Hedger J., Degradation of cell wall polymers. In: Fungi in bioremediation, Cambridge;. Gadd G. Ed Cambridge University Press., U.K.,2001.

[9] Sasek V., Why mycoremediations have not yet come to practice. In Sasek V. *et al.,* (Eds.) In: The utilization of bioremediation to reduce soil contamination: Problems and solutions, Kluwer Academis Publishers. 2003; 247-276

[10] Johal G.S. and Huber D.M,. Glyphosate effects on diseases of plants, *Eur. J. Agron.*, 31 (2009) 144-152.

[11] Mbanaso F.U., Coupe S.J., Charlesworth S.M. and Nnadi E.O., Laboratory-based experiments to investigate the impact of glyphosate-containing herbicide on pollution attenuation and biodegradation in a model pervious paving system, *Chemosphere*, 90 (2013) 737-746.

[12] Shushkova T., Ermakova I. and Leontievsky A., Glyphosate bioavailability in soil, *Biodegradation*, 21 (2010) 403-410.

[13] Solomon K.R., Anadón A., Carrasquilla G. and Cerdeira A.L., Coca and poppy eradication in Colombia: environmental and human health assessment of aerially applied glyphosate, *Rev. Environ. Contam. Toxicol.*, 190 (2007) 43-125.

[14] Leckie S.E., Methods of microbial community profiling and their application to forest soils, *Forest Ecol. Manag.*, 220 (2005) 88-106.

[15] Kryuchkova Y.V., Burygin G.L., Gogoleva N.E. and Gogolev Y.V., Isolation and characterization of a glyphosate-degrading rhizosphere strain, *Enterobacter cloacae* K7. *Microbiol. Res.*, 169 (2014) 99-105.

[16] Qiao X., Ma, L., and Hummel H.E. Persistence of atrazine and occurrence of its primary metabolites in three soils, *Journal of Agriculture and Food Chemistry*, 14 (1996) 187-192.

[17] Kruger E.L, Rice P.J., Anhalt J.C., Anderson T.A. and Coats, J.R. Use ofundisturbed soil columns under controlled conditions to study the fate of [^{14}C] diethylatrazine, *Journal of Agriculture and Food Chemistry*, 44 (1996) 1144-1149.

[18] Ayansina A.D.V. and Oso B.A., Effect of two commonly used herbicides on soil microflora at two different concentrations, *African Journal of Biotechnology*, 5 (2) (2006) 129-4132.

[19] Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. and Williams S.T., Bergye's Manual of

Determinative Bacteriology, 9th Ed. Baltimore, Maryland: Williams and Wilkins, 1994.

[20] Cheesbrough M., Medical Laboratory Manual for Tropical Countries, Microbiology. Linacre House, Jordan Hill, Oxford. Vol.11., (1984).

[21] Moneke A.N., Okpala G.N. and Anyanwu C.U., Biodegradation of glyphosate herbicide in vitro using bacterial isolates from four rice fields, *African Journal of Biotechnology*, 9 (26) (2010) 4067-4074.

[22] Diez M.C., Biological aspects involved in the degradation of organic pollutants, *J. Soil. Sci. Plant Nutrition*, 10(3) (2010) 244-267.

[23] Chirnside A., Ritter W. and Radosevich M., Isolation of selected microbial consortium from a pesticide-contaminated mixed load site soil capable of degrading the herbicides atrazine and alachlor, *Soil Biology and Biochemistry*, 39 (2007) 3056-3065.

[24] Whitelaw-Weckert M., Hutton R., Rouse E. and Lamont R., The effect of herbicides on permanent swards on soil microbial populations, In 3rd Australian New Zealand Soils Conference, University of Sydney, Australia, (2004) 1-5.

[25] Filimon M.N., Popescu R., Borozan A.B., Bordean D.M., Dumitrescu G., and Voia S.O., Influence of xenobiotic substances on actinomycete communities in soil, *Animal Science and Biotechnologies*, 45(2) (2012) 221-224.

[26] Lynch J.M., Microorganisms and enzymes in the soil. In: Soil Biotechnology, Microbial Factors in Crop Productivity, London: Blackwell Sci. Publ., ,1983; 185.

[27] Radosevich M., Traina S.J., Hao Y.I. and Touvinen O.H., Degradation and mineralization of atrazine by a soil bacterial isolate, *Applied and Environmental Microbiology*, 61 (1995) 297-302.

[28] Milosevic N.A. and Govedarica M.M., Effect of herbicides on microbiological properties of soil, *Proceedings for Natural Sciences*, *Matica Srpska Novi Sad.*, 102 (2002) 5-21.

[29] Singh B.K., and Walker A., Microbial degradation of organophosphorus compounds, *FEMS Microbiological Review*, 30 (2006) 428-471.

[30] Lipok J., Dombrovska L., Wieczorek P. and Kafarski P., The ability of fungi isolated from stored carrot seeds to degrade organophosphonate herbicides: in pesticides in air, plant, soil and water system, *Proceedings of the XII Symposium Pesticide Chemistry*, (2003).

[31] Dibua U.M.E., Mkpuma V. O. and Enemuo S., Isolation, Characterization and Biodegradation Assay of Glyphosate Utilizing Bacteria from Exposed Rice Farm, *Journal of Biology, Agriculture and Healthcare*, 5(5) (2015) 96-109.

[32] Adelowo F.E., Olu-Arotiowa O.A. and Amuda O.S., Biodegradation of Glyphosate by Fungi Species, *Advances in Bioscience and Bioengineering*, 2(1) (2014) 104-118.

[33] Martins P.F., Martinez C.O., Giselle de Carvalho Carneiro P.I.B., Azevedo R.A., Pileggi S.A.V. and Itamar Soares de Melo, Pileggi, M., Selection of microorganisms degrading S-Metolachlor herbicide, *Brazilian Archives of Biology and Technology*, 50(1) (2007) 153-159.

[34] Ferrey M.L., Koskinen W.C., Blanchette R.A. and Burnes T.A., Mineralisation of Alachlor by lignin-degrading fungi, *Canadian Journal of Microbiology*, 40 (9) (1994) 795-798.