BACTERIAL EMPIRE

2022, VOL. 5, NO. 4, e401

OPEN ACCESS

SCICELL

BIODEGRADATION OF GLYPHOSATE CONTAINING HERBICIDE BY SOIL BACTERIA

OMORINOLA ALAKE A.[1,2]*, OGUNBIYI OLUWAGBENGA J.*[2] *& OWOH-ETETE UKAMAKA*[2]

Address (es):
[1]Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria
[2]Biology Unit, Faculty of Science, Air Force Institute of Technology, P.M.B. 2104, Kaduna, Nigeria

*Corresponding author: alakeomorinola@gmail.com

https://doi.org/10.36547/be.401

ABSTRACT

Sixty bacterial isolates were isolated from two herbicide contaminated soil farms (Amoyo and University of Ilorin Nursery section both in Kwara State) after enrichment with mineral salt medium (MSM) supplemented with glyphosate containing herbicide. The organophosphorus herbicide efficiently stimulated the growth of bacterial isolates. Bacteria isolated were subjected to screening using varying concentrations of the herbicide ranging from 0.1 to 3% prepared with mineral salt medium. This screening revealed that all the bacterial isolates had biodegradative potential. They however had varying degradative potentials as some had heavy growth while others had only slight growth or no growth on increased concentrations of herbicide. Screening reduces the bacterial populations with growth ranging from 5.0 x 10^4 to 2.33 x 10^8 while the number of bacterial isolates reduced from sixty to thirteen. These bacterial isolates were *Pseudomonas putida*, *Bacillus safenis*, *Lysinibacillus fusiformis*, *Micrococcus* sp., *Arthrobacter* sp., *Aeromonas* sp., *Bacillus cereus*, *Flavobacterium* sp., *Acinetobacter rhizoshaerae*, *Bacillus pumilus*, *Achrobacterium* sp., *Bacillus* sp., and *Ochrobacterium* sp. The increase in viable bacterial counts over the period of 21 days indicated the use of herbicide as carbon source for their various metabolism processes. These bacteria can be recommended as environmentally safer alternatives tools to protect soil from excessive use of glyphosate containing herbicide residues which might contaminate the soil.

Keywords: bacteria, isolates, herbicide, glyphosate, biodegradation

INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum systemic herbicide used to kill weeds, especially annual broadleaf weeds and grasses known to compete with commercial crops grown around the globe. A study on mice has found that a single intraperitoneal injection of Roundup in concentration of 25 mg/kg caused chromosomal aberrations and induction of micronuclei (**Stephen *et al.*, 2008**; **Prasad *et al.*, 2009**). A study of various formulations of glyphosate found that risk assessments based on estimated and measured concentrations of glyphosate that would result from its use for the control of undesirable plants in wetlands and over-water situations showed that the risk to aquatic organisms is negligible or small at application rates less than 4 kg/ha and only slightly greater at application rates of 8 kg/ha (**Solomon *et al.*, 2003**). Glyphosate formulations are much more toxic for amphibians and fish than glyphosate alone (**Salbego *et al.*, 2010**). Aquaculture, freshwater and marine fisheries supply about 10% of world human calorie intake (**Relyea, 2005**).
Bacteria strains capable of utilizing methyphosphonic acid (MP) or glyphosate (GP) as the sole source of phosphorus were isolated from soils contaminated with these organophosphonates. Strains *Achromobacter* sp. MPS (VKM B-2694), MP degraders group and *Ochrobactrum anthropi* GPK 3 (VKM B-2554D), GP degraders group, demonstrated the best degradative capabilities towards MP and GP, respectively and were studied for the distribution of their organophosphate catabolism systems (**Sviridov *et al.*, 2012**).
Herbicides have been reported to have widely variable toxicities. Some herbicides can cause a range of health effects, ranging from skin rashes to death (**Gilbert and Scott, 2010**). In addition to the risk of herbicide pollution to ecosystems and the risks to human health, the residues of herbicides used on previous crops can cause phytotoxicity to the rotation crops, threaten the safety of food production (**Kinney *et al.*, 2004**). Due to their highly toxic nature and long persistence in the environment, there is considerable concern regarding herbicide residues in ecosystems.
Herbicides are extraneous to soil component pools and are expected to affect the catalytic efficiency, behavior of soil enzymes (**Bollag and Liu, 1990**; **Sannino and Gianfreda, 2001**), which contribute to the total biological activity of the soil-plant environment under different states (**Dick, 1994**). The interaction between herbicides and soil microorganisms may be of practical significance because of possible inhibition in microbial activities contributing to soil fertility. Various studies have revealed that the herbicides can cause qualitative and quantitative change in enzyme activity (**Sannino and Gianfreda, 2001**; **Saeki and Toyota, 2004**; **Sebiomo *et al.*, 2011**, **Xiao *et al.*, 2011**). Hence, the effects of herbicides on soil microbial communities addressing the apprehensions about the environmental impacts of herbicide use. However, the aim of this study is to exploit the ability of microorganism to degrade herbicide residue from agricultural soils.

MATERIALS AND METHODS

Sterilization of Glassware and other materials

All glassware used were thoroughly washed with detergent, rinsed and allowed to dry. The glassware was then wrapped with aluminium foil and sterilized in the hot air oven at 170ºC for 60 minutes. The distilled water used for serial dilution, was autoclaved at 121ºC for 15 minutes. The work bench was swabbed with 70% alcohol before and after every experiment.

Sample site

Two agricultural sites contaminated with herbicide were selected; these were farms from Amoyo in Ifelodun Local Government Area of Kwara State (Latitude 8.63°N and Longitude 5.18°E) and Nursery Section of the Department of Forestry, University of Ilorin, Kwara State (Latitude 8.4799°N and Longitude 4.5418°E). At each location, four (4) different samples were collected.

Soil sampling

Two soil samples were collected at four different points at each location and were labeled A1, A2, A3, A4 and B1, B2, B3 and B4 respectively. At each point, soil samples were collected randomly 5-10 cm beneath the surface of the soil using sterile hand trowel and were packed in sterile polythene bags properly labeled. The samples were immediately transferred to Microbiology laboratory for analysis.

Herbicide

Two brands of herbicides containing one active ingredient which is glyphosate were used in this study. The brands were Forceup and Goldensate with the active ingredient of analytical standard glyphosate (44.1%) which was provided by an Agro chemical shop in Ilorin metropolis, Kwara State.

Physicochemical Analysis of Soil Samples

The physicochemical characteristics of the soil samples that were determined include: temperature, pH, percentage moisture content and organic matter content. Soil pH, percentage moisture content and organic matter content were determined based on the method describe by Pramer and Schmidt (**1964**).

Preparation of Stock Mineral Salt Medium for Bacteria

Stocks were prepared inside plastic bottles with sterile distilled water using the following composition of mineral salts as described by Eman *et al.* (**2013**). The constituents for stock mineral salt medium for bacteria were:

Bacteria (litre)	For 150 ml
10g NaCl	15ml of 10% NaCl
0.42g $MgSO_4.7H_2O$	3.15ml of 2% $MgSO_4.7H_2O$
0.29g KCl	2.18ml of 2% KCl
1.25g K_2HPO_4	9.38ml of 2% K_2HPO_4
0.83g KH_2PO_4	6.23ml of 2% KH_2PO_4
0.42g $NaNO_3$	3.15ml of 2% $NaNO_3$
Herbicide	1/2/3ml of Forceup/Goldensate
15g Agar-agar	2.3g of Agar powder

Preparation of Media

All these mineral salts were measured into different plastic bottles and dissolved with 100 ml distilled water as stock. Calculated volume of each mineral salts were taken from various stocks with sterile pipettes, in order to make 150 ml solution, 100 ml distilled water and 20 ml tap water was added as a source of trace elements to make up the 150 ml, followed by 2.3 g of Agar-agar. The mixture was heated, homogenized and autoclaved at 121ºC for 15 minutes. The volume of each stock solution used for 150 ml mineral salt agar medium for fungi and bacteria is described earlier above.

Microbiological Analysis of Soil Samples

Isolation of Bacteria from Soil Samples

This was done using the soil dilution pour-plate method as described by Eman *et al.* (**2013**).

Screening of Bacterial Isolates for Biodegradative Potential

Screening of bacterial isolates and growth capability on herbicide mineral salt agar medium was done qualitatively following the method described by Eman *et al.* (**2013**). Bacterial screening was carried out by monitoring the growth capability on herbicide containing mineral salt media at different concentrations (0.1%, 1%, 2% and 3%).

Purification and Preservation of Bacterial Isolates

Pure cultures of bacteria were isolated by using a sterile wire loop to pick a representative colony from the parent plate. Subculture was done on a solidified sterile nutrient agar plate by streaking under aseptic condition. The plates were incubated upside down at 37ºC for 24 hours. The pure cultures obtained were thereafter transferred into separate sterile nutrient agar slant, incubated at 37ºC for 24 hours labeled and stored in the refrigerator at 4-8ºC as stock cultures.

Characterization and Identification of Bacterial Isolates

The characterization and identification of bacterial isolates were based on the colonial morphology, cellular characteristics and biochemical tests carried out on pure culture of isolates (**Buchanan and Gibbons, 1974**; **Fawole and Oso, 2001**; **Dubey and Maheshwari, 2004**; **Garrity *et al.*, 2004**).

Colonial Morphology

The colonial morphology of the bacteria was based on the shape, size, elevation, edge, transparency and pigmentation of the isolates.

Cellular Characteristics

This involves staining and microscopic examination of the bacterial isolates. The cellular characteristics include Gram staining, spore staining and motility test. All these cellular characteristics were determined as described by Fawole and Oso (**2001**).

Biochemical Test

Biochemical test such as catalase test, indole test, Voges-Proskauer (VP) test, citrate utilization test, oxidase test was carried out according to the method described by Fawole and Oso (**2001**) while urease test and methyl red test were carried out following the method described by Brock *et al*. (**1994**) and Olutiola *et al*. (**1991**) respectively.

Molecular Identification

All the selected isolates were inoculated in sterile nutrient broth which was at 37ºC incubated for 24 hours and then transferred to International Institute of Tropical Agriculture Laboratory, Ibadan, Oyo State, Nigeria for further identification using molecular technique.

Molecular Characterization of Bacterial Isolates

Molecular characterization was done using 16s rRNA polymerase chain reaction (PCR) techniques, bacterial isolates was further identified at genomic level.

Isolation of Genomic DNA

The genomic DNA of the strain was isolated according to the instruction described in QIAamp DNA mini kit (250) cat no 51306.

PCR Amplification of 16rRNA region

PCR amplification was done to confirm the identity of the bacterial strain. To get an amplification size of 1500bp, the small sub-units 16s rRNA genes were amplified from the genomic DNA with 16sF (5' AGAGTTTGATCCTGGCTCAG 3) and 16sR (5' TACCTTGTTACGATT 3') primers. Amplification were carried out in 20 µl reaction mixture consisting of 10 x buffer 2.0 µl: 2.0mMdNTPs; 2.0 µl; 3.0 U/µl taq DNA polymerase, 0.2 µl; 100 ng/µl of each primer, 1.0 µl; template DNA, 1.0 µl and sterilized distilled water 12.8 µl in a (Biorad, USA) thermal cycler using the PCR conditions 95ºC for 2 minutes (denaturation), 53.8ºC (strain R5), 52.3ºC (strain k22) and 60.40ºC (strain N11) for 1 minute (annealing) and 72ºC for 3 minutes (extension). The total number of cycles was 40, with the final extension of 72ºC for 10 minutes. The amplified product (50 µl) were size separated on 1% agarose gel prepared in 1% TAE buffer containing 0.5 µg/ml ethidium bromide and photographed with the gel documentation system (Biored, USA). A 100bp DNA ladder (Genei) was used as molecular weight size markers.

Purification of the PCR product

The PCR product (1500bp) was purified from contaminating products by electroelution of the gel slice containing the excised desired fragment with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µl of nuclease free water.

Nucleotide sequencing

Sequencing pattern- the PCR amplicons obtained by amplifying PCR products was diluted in Tris buffer (10 mM, pH 8.5). The dilution used was 1:1000 in order to obtain the DNA concentration required 8 µl DNA. The primers used in all sequencing reactions was 16sF (5' AGAGTTGATCCTGGCTCAG 3') at a concentration of 3 µM. Sequencing was then performed using an Automated Sequencer (ABI PRISM 310, Applied Biosystem, USA).

Bacterial Biodegradation study

The composition of the mineral salt broth medium used for bacterial degradation study was the same composition of stock mineral salt medium for bacteria with the exemption of agar-agar. Four hundred milliliters of the medium were prepared in 500ml conical flask and 6ml of the test herbicide was added as source of carbon and energy before the medium was sterilized in an autoclaved at 121ºC for 15 minutes. The progress of degradation study was assessed by measuring the change in pH, optical density and changes in bacterial population over the period of time. Changes in viable bacterial count were determined according to Okerentugba and Ezeronye (**2003**). Changes in pH of the mineral salts broth were monitored and changes in optical density of the mineral salts broth were measured spectrophotometrically by following the method described by Ekundayo and Osunla (**2013**).

RESULTS

Physicochemical Parameters of Herbicide Contaminated Soil Sample

The physicochemical parameters of herbicide-contaminated soil considered include: percentage of moisture content, organic matter content and pH. The results of the physicochemical parameters tested from eight soil samples as were presented in Table 1. The soil colour ranges from gray to black. The percentage moisture content ranged from 32 to 50%. The percentage organic matter content of the soil samples ranged from 47.81 to 64.06%, while the pH of the soil samples ranged from 6.20 to 8.00 as shown in Table 1.

Table 1 Physicochemical Parameters of Soil Samples

Sample	*Colour*	*Temperature ℃*	*pH*	*Moisture Content (%)*	*Organic matter (%)*
A1	Black	30.2	6.26	40	47.81
A2	Grey	30.5	7.56	37	48.69
A3	Grey	31.0	7.56	42	50.03
A4	Black	32.4	6.55	50	48.20
B1	Dark Grey	30.3	6.43	32	58.60
B2	Black	30.7	6.44	36	62.51
B3	Grey	30.5	7.42	38	64.06
B4	Black	31.1	8.0	36	60.67

Key: A= A farm in Amoyo; B= A farm in Nursery Section at Unilorin

Isolation and Characterization of Bacteria Isolates

A total number of sixty for Force up brand and forty-one for Goldensate brand bacterial isolates were isolated from the soil using mineral salt medium. These isolates were subjected to screening with different concentrations of herbicide ranging from 0.1 to 3% herbicide concentrations. This reduces the species to a total number of eight (8) for Force up and five (5) for Goldensate.

Bacterial isolates were identified using the manuals of Buchanan and Gibbons (**1974**) and Cullimore (**2009**), which were identified as *Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp., *Arthrobacter* sp., *Acinetobacter* sp., *Achrobacterium* sp., and *Ochrobacterium* sp. These were further subjected to 3% concentration screening which reduces the bacterial isolates to six species for Force up taking them longer time (78 hr) to grow on the medium. These species include *Lysinibacillus fusiformis*, *Bacillus safensis*, *Bacillus pumilus*, *Bacillus cereus*, *Ochrobacterium anthropi* and *Arthrobacter* sp., and two for Goldensate, which are *Flavobacterium* sp., and *Bacillus* sp., with bacteria count ranging from 1.0 x 10^3 – 3.6 x 10^4 cfu/ml.

Table 2 Growth of bacterial isolates in mineral salt agar medium (MSM) supplemented with 2% and 3% (v/v) of Force up and Goldensate herbicides brands

S/N	*Bacterial Isolates*	*Degree of growth in 2% MSA*	*Degree of growth in 3% MSA*
	Force up		
1	*Lysinibacillus fusiformis*	++++	++++
2	*Bacillus safensis*	++++	++++
3	*Bacillus pumilus*	++++	++++
4	*Bacillus cereus*	++++	++++
5	*Arthrobacter* sp.	++	+
6	*Aeromonas* sp.	+	-
7	*Acinetobacter rhizoshaerae*	++	-
8	*Ochrobacterium anthropi*	+++	++
	Goldensate		
1	*Achrobacterium* sp.	+++	-
2	*Flavobacterium* sp.	+++	++
3	*Bacillus* sp.	++	+
4	*Micrococcus* sp.	++	-
5	*Pseudomonas putida*	+++	-

Keys:
+++++: Profused growth
++++: Heavy growth
+++: Moderate growth
++: Slight growth
+: Very slight/faint growth
-: No growth

Lysinibacillus fusiformis, *Bacillus safensis*, *Bacillus pumilus* and *Bacillus cereus* were selected for biodegradability study due to their high biodegradative potential on MSA supplemented with 3% of Force up- herbicide. Details of growth of all biodegrading bacterial isolates were shown in Table 2. However, only Force up herbicide was used for the biodegradative study due to the biodegradative potential of the microorganism on the mineral salts medium agar supplemented with Force up herbicide and it is the most used. Details of the degree of growth of all the biodegrading bacterial isolates on MSA were also shown in Table 2. The colonial morphology, cellular characteristics and biochemical tests of bacteria isolates were shown and presented in Table 3. The following

Table 3 Characterization and Identification of Bacterial Isolates

Cellular characteristics

Shape of colony	Size	Optical characteristics	Colour	Spore strain	Cell shape	Cell arrangement	Gram reaction	Motility	Oxidase	Catalase	Indole	Vogues proskauer	Methyl red	Citrate	Urease	Starch hydrolysis	Oxidative fermentation	Slope	Butt	Gas
I	Small	T	Cream	+	R	Single	+	-	+	+	-	+	-	-	-	+	Fe	K	K	-
I	Small	T	Cream	+	R	Single	+	-	+	+	-	+	-	-	-	+	Fe	K	K	-
I	Small	Op	Green	-	R	Cluster	-	+	+	+	-	-	-	+	-	+	FA	A	A	-
C	Small	Op	Orange	-	R	Cluster	-	+	+	+	-	-	-	-	-	+	Fe	K	K	-
C	Medium	Op	Cream	-	R	Single	-	-	-	+	-	-	+	+	-	-	Ae	K	A	-
I	Small	T	Cream	+	I	Single	+	-	+	+	+	+	-	-	-	+	Fe	K	K	-
I	Small	T	Cream	+	R	Single	+	-	+	+	+	+	-	-	-	+	Fe	K	K	-
C	Small	Op	Cream	-	Co	Single	+	-	+	+	-	+	-	-	+	-	Fe	A	A	-
C	Large	Tr	Cream	+	R	Pair	-	-	-	+	-	+	-	-	+	-	Ae	A	A	-
C	Small	Op	Yellow	-	R	Single	-	-	+	+	-	-	-	+	-	-	Fe	K	A	-
C	Large	Op	Yellow	-	R	Single	-	+	+	+	-	-	-	+	+	-	Ae	K	A	-

Keys:
R: Rod; I: Irregular; Op: Opaque; T: Transparent; Tr: Transluscent; K: Alkaline; A: Acid; AG: Acid and gas production; C: Circular; Co: Cocci; FA: Facultative anaerobe; Fe: Facultative aerobe; Ae: Obligate aerobe; +: Positive; -: Negative.

Screening of Bacterial Isolates in Mineral Salts Agar (MSA)

Lysinibacillus fusiformis RB-21, *Bacillus safensis* U17-1, *Bacillus pumilus* GLB197 and *Bacillus cereus* M3 grew heavily, *Ochrobacterium anthropi* grew moderately while *Arthrobacter* sp., *Aeromonas* sp., and *Acinetobacter rhisoshaerae* grew slightly on MSA plates at 2% concentration of Force up herbicide. *L. fusiformis* RB-21, *B. safensis* U17-1, *B. pumilus* GLB197 and *B. cereus* M3 grew heavily on MSA plates at 3% concentration of herbicides (Force up) while *O. anthropi* grew slightly, taken them 3 to 5 days to grow on their various plates. *Aeromonas* sp. and *Acinetobacter rhizoshaerae* showed no growth on MSA plates.
Moreover, *Achrobacterium* sp., *Flavobacterium* sp., and *Pseudomonas* sp. grew heavily on 2% concentration of Goldensate herbicide while Bacillus sp. and Micrococcus sp. grew slightly. However, only *Flavobacterium* sp. and *Bacillus* sp. grew slightly while *Achrobacterium* sp., *Micrococcus* sp. and *Pseudomonas putida* showed no growth on MSA plates at 3% concentration of Goldensate herbicide. Details of growth of all biodegrading bacterial isolates on MSA plates are shown in Table 2.0.

Molecular Identification

Molecular characteristics using sRNA PCR technique further confirm the observation of the morphological and biochemical identification of the bacterial isolates in this study. The results conform to the four bacterial isolates used for the biodegradation study on the study. Figure 1-4 shows the nucleotide for *Lysinibacillus fusiformis* strain RB-21 as F1; *Bacillus safensis* strain U17-1 as F2; *Bacillus pumilus* strain GLB197 as F3 and *Bacillus cereus* strain M3 as F4. However, the results of the molecular examination revealed all the isolates sent to be *Bacillus* with different species and these were used for biodegradation study.

F1
CTCTGATCGGATATTGGGCGTAAGCGCGCGCAGGTGGTTTETTAAGTT
CTGATGTGA
AAACCCACAGGCTCAACTTCGGATCGTCCTTGGAAACTGGGAAACITG
AGTGCAAA
AAAGGATAGTGAAATTCCCAGTGTACGGGTGAAATGCGTAAAGATTT
GGAGGAAC
ACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACCETGAAGGCG
CGAAAGCGG
GGGGAGCAAACAGAATTAAATACCCTGGTAGTCCACGCCGTAAACAA
TGATGGCT
AAGTGTTAGGGTTTTCCGCCCCCCTTAGTGCTGAAGCTAACEGCATTA
AGCATTCCGCC
GGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGATTTGAAGGGGAC
CCCGCACAA
GCGGGGAAGTATGTGGTTTATTTCGAAGCAACGGAAGAACCTTACCA
GGTCTTGA
CATCCCGTTGAACCCTGTAGAAATATAGGTTECCETTEGGAGGGAACT
GTCACAGGT
GGCACGGGGTTGTCATCTCTCGTGTCGAGATGTTGTGGTTTAACTCGC
GCACGAGC
GCACTCGAGATCAGATGTCGCCACATTTAGTTGGGCTCCTATGTEGAC
TGCGGTAAA
ACCGGAGAAGGGGGGGAGACTCAAATATCAGGCCCTTAAGTTCGGCG
TAGAATCGC
TATATGGTGTAGACACGGTGCCACTCCAGTGGGACCTATCAACGAGC
CGTCTCATTC
GAATGAAGCGCACTCGCTGCTAATCGGATCTATATEGGGATOCAGCTG
CCGTAACTC
CCCGGTCTAAA
Figure 1 DNA Sequence of *Lysinibacillus fusiformis* RB-21.

F2
GGCGCGTCGGATATTGGGCGTAAGGGCTCGCAGGCGGTTICTTAAGTC
TGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTIG
AGTGCAGA
AAAGGAGAGTGGAATTCCACGTGTAGGGGTGAAATGCGTAAAGAATG
TGGAGGAAC
ACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGEGAG
CGAAAGCGTG
GGGAGCGAACAGGATTAAATACCCTGGTAGTCCACGCCCGTAAACGA
TGAGGGCTA
GGTGTTAGGGGTTTCCGCCCCCCTTAGGGCTGAAGTTAAACGCATTAA
GCATTCCGCC
GGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTTGACGGGGG
CCGCACAA
GCGGTGAAGCATGTGGTTTAATTCGAAGCAACGCCGAAAAACCTTAC
CAGGTCTTGA
CATOCTCTGACAACCCTAAAGATAGGGGTTTECCTTCCGGGAACAGAG
TGACAGGTG
GTGCAGGGTTGTCGCCACCTCGTGCCGTGAGATGTTGGGTTAAGTCCC
GCAACGAG
CGCAACCCTTGATETTAGTTGCCAGCATTCAGTTGGOGCCCCCTAAGG
TGACTGCCGG
TGAÁAAACCGGAGGAAGGTGGGGATGACCTCAAATCTTCATGCCCCT
TATGACCTG
GGCTAGACACTTGCTACTATGGACTGAACAAGGGTTGOCCAGACTGC
AAGGTGGAGC
CCATCCCAAAAATCTGGTCTCTCATCTGGATAGTAAACCAGCACTCCG
CTGCCGCGA
GTCTGCGAACTETTTCAGACGEGGATCAGCACGCCGCCGGTGATACGC
CTCCCCCG
GGCCTCCA
Figure 2 DNA Sequence of *Bacillus safensis* U17-1.

F3
AGGCTGTCGGATATTGGGCGTAAGGGCTCGCAGGGGTTTOCTTAAGTC
TGATGTGA
AAGCCCCCGGCTCAACCGGGGGAGGTCATTGGAAACTGGGAAACTTG
AGTGCAGA
AGAGGAGAGTGGAATTCCACGTGTAGGGTGAAATGCGTACGAGATGT
GGAGGAAC
ACCAGTGGCGAAGGGACTCTCTGGTCTGTAACTGACGCTGAGGAGCG
AAAGCGTG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAACGA
TGAGTGCTA
AGTGTTAGGGGTTTCCGCCCCCTTAGTGCTGCAGCTAACCGCATTAAG
CACTCCGCCT
GGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTOGACGGGGG
CCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGGAAGAACCTTACCAG
GTCTTGAC
ATCCTCTGACAACCCTAGAGATAGGGETTTCCCTTCGCGGGACAGAGT
GACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTGTGAGATGTTGGGTTAAGTCCCGC
AACGAGC
GCAACCCTTGATETTAGTTGCCAGCATTTAGTTGGGCAACTCTAAGGT
GACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGG
GCTACACACGTGCTACAATGGACAGAACAAAGGGGGTOGCGAGACCG
CAAGGTTTAG
CCCATCTCACAAATETGTTCTCAGTTCGGATCGGAGTETGCAACTCGA
CTGCGTGAA
GCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACTTC
CCCGGGGG
TCTAAAA
Figure 3: DNA Sequence of *Bacillus pumilus* GLB197.

F4
AGCGGCACGGATATTGGGCGTAAGCGGGGGGGGGTGGTTTCTTAAGT
CTGATGTGA
AAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAACTT
GAGTGCAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGGTAGAAGATAT
GGAGGAAC
ACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAAGGCG
CGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCGTAAACGA
TGAGTGCTA

AGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACCGCATTAAG
CACTCCGCCT
GGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTOGACGGGGG
CCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGGAAGAAACCTTACCA
GGTCTTGAC
ATCCTCTGACAACCCTAGAGATAGGGETTETECTTEGGGGAGCAGAGT
GACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTGTGAGATGTTGGGTTTAAGTCCCG
CAACGAGC
GCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTTCTAAGGT
GACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCT
TATGACCTGG
GCTACACACGTGCTACAATGGACGGTACAAGAGETGCAAGACCGCGA
GGTGGAG
CTAATCTCATAAAACCGTTCTCAGTTEGGATTGTAGGCTGCCAACTCG
CCTÁCGTGAA
GCTGGAATCGCTAGTAATCGGGATCAGCATGCCGGGGTTGAATACTTC
CCCGGGT
CCACAAA

Figure 4: DNA Sequence of *Bacillus cereus* M3.

Bacterial Biodegradation Studies

Changes in Bacterial Population during Biodegradation

Changes in bacterial population in mineral salt medium broth (MSB) supplemented with 2% (v/v) Force up- herbicide during biodegradation. Viable counts of the biodegrading bacterial isolates for *Lysinibacillus fusiformis* RB-21 the ranged was between 5.0 x 10^4 and 2.33 x 10^8. For *Bacillus safensis* U17-1 the ranged was between 2.3 x 10^5 and 2.26 x 10^6. For *Bacillus pumilus* GLB197 the ranged was between 1.1 x 10^5 and 7.9 x 10^6 and for *Bacillus cereus* M3 the ranged was between 6.8 x 10^5 and 2.80 x 10^6. During the 21-days period of biodegradation, the peak values generally tend to be on day 6 for all isolates, after which the viable counts starts falling. Details of the viable counts for each isolates are shown in Figures 5-8.

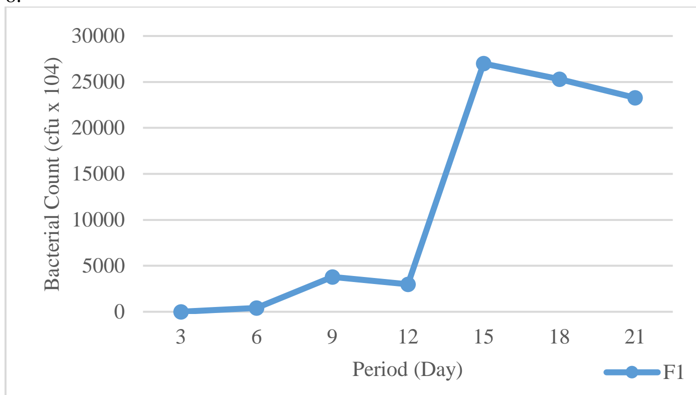

Figure 5 Changes in the population of *Lysinibacillus fusiformis* RB-21 in Mineral Salts Broth supplemented with 2% of force up- herbicide

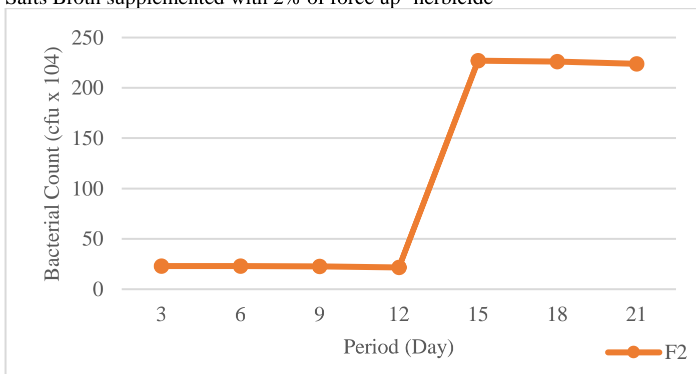

Figure 6 Changes in the population of *Bacillus safenis* U17-1 in the Mineral Salts Broth supplemented with 2% of force up- herbicide

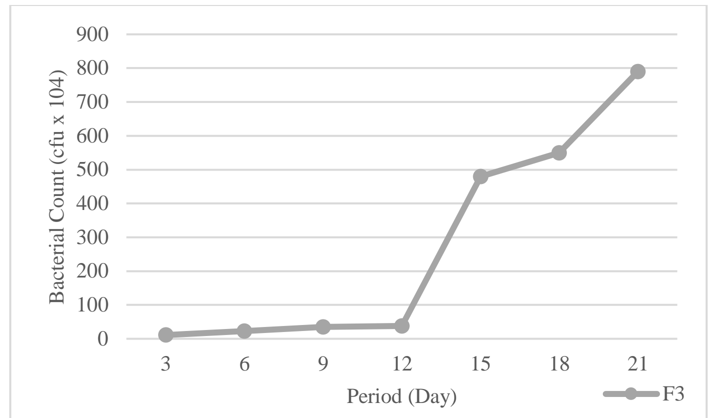

Figure 7 Changes in the population of *Bacillus pumilus* GLB197 in the Mineral Salts Broth supplemented with 2% of force up- herbicide

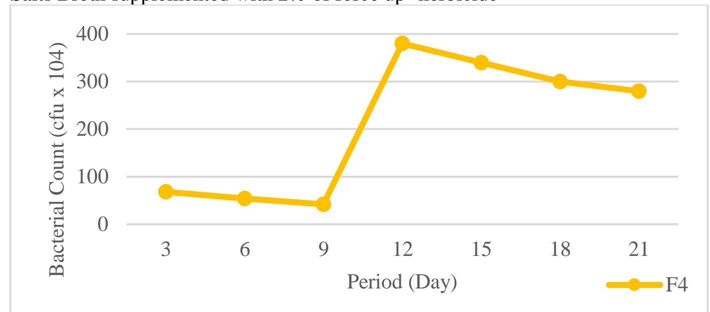

Figure 8 Changes in the population of *Bacillus cereus* M3 in the Mineral Salts Broth supplemented with 2% of force up- herbicide

Changes in pH of MSB during Biodegradation

The pH values obtained during the course of the biodegradative study ranged from 6.60 to 6.97 for *Lysinibacillus fusiformis* RB-21; 6.60 to 7.2 for *Bacillus safensis* U17-1; 5.00 to 6.00 for *Bacillus pumilus* GLB197 and between 5.00 and 6.400 for *Bacillus cereus* M3; details of changes in pH values are shown in Figure 9.

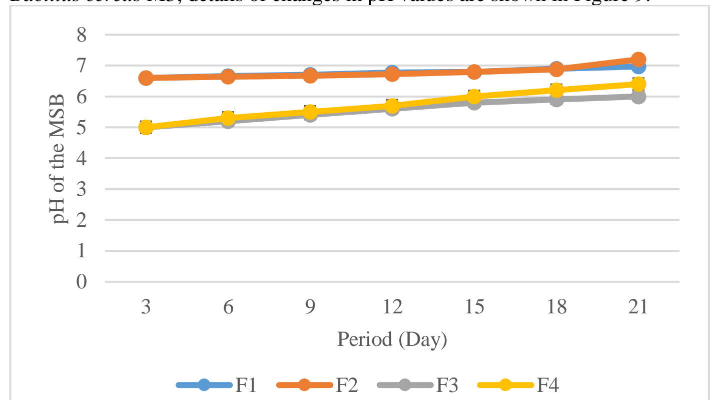

Figure 9 Changes in pH of Mineral Salts Broth supplemented with 2% force up-herbicide during bacterial biodegradation

Changes in optical density (A@600nm) of MSB during Biodegradation

Values obtained for changes in optical density of MSB ranged from 0.018 to 0.026 for *Lysinibacillus fusiformis* RB-21; 0.009 to 0.029 for *Bacillus safensis* U17-1; 0.014 to 0.026 for *Bacillus pumilus* GLB197 and 0.037 to 0.18 for *Bacillus cereus* M3. Details of the changes in optical density are shown in Figure 10.

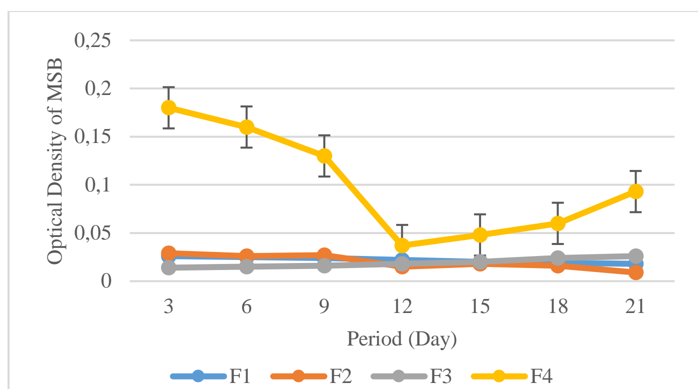

Figure 10 Changes in Optical Density of the Mineral Salts Broth with bacterial isolates during bacterial biodegradation

DISCUSSION

Results obtained from the physicochemical parameters of the herbicide contaminated soils sampled during this research indicate that pH of the soil samples generally ranged from 6.26 to 8.00. This ranged is quite close to neutrality and would permit growth of a wide variety of microbial species. This result is similar to that of Rohilla and Salar (**2012**) who reported that a pH range of 6.20 to 8.50 is required for optimal microbial activity. Moisture contents of the soil samples ranged from 32 to 42% whereas Frazar (**2000**) reported that an optimum moisture content of 30 to 90% is required for biodegradation. The moisture content of the soils contaminated with herbicide can be attributed to the seasonal factor as sampling was done during rainy season. It could also be due to anthropological factors as these sites were agricultural farms and irrigation is being carried out to supplement rainfall. The colour of soil samples ranges from brown to black, with variation in pH between 6.20 and 8.00. The temperature of the soil was high (30.2 – 31.1ºC) with great variation in percent moisture content, organic matter ranges from 47.81 to 60.67%. The results revealed that the soil samples obtained from various sites shown heterogeneity in physicochemical parameters. Soil properties like organic matter and moisture content affect the density and diversity of microbes in the soil (**Rohilla and Salar, 2012**). The moisture content in soil acts as solvent and is essential for microbial functioning. A certain minimum level of organic matter and moisture content is essential to ensure the presence of an active microbial population in the soil.

Microbial analysis of herbicide contaminated soils yielded viable bacterial counts for all bacterial isolates used ranging from 2.33 x 10^8 to 1.1 x 10^5. These bacterial populations were generally more in soil than fungal population as reported by Baxter and Cummings (**2008**). Bacterial isolates from herbicide contaminated soils are known to be indigenous soil flora. Herbicide contaminated soils would have altered characteristics and population of microorganisms when compared to uncontaminated soils. The chief difference would be in the amount of oxygen and microbial population due to the fact that oxygen would be used up during the process of degrading the herbicide and the metabolites released could lyse other microorganism which decreases microbial population. However, some of these microbes might not have the ability to grow in the presence of these xenobiotic chemicals used for herbicide.

The principle of biodegradation is based on the premise that the biodegrading organism is capable of utilizing the chemical compound, in this case, herbicide as a source of carbon. The herbicide then becomes the carbon source that fuels the organisms metabolism and as the organism carries out it metabolic processes, the herbicide is metabolized into products with relatively lesser toxicity or totally harmless products.

Biodegrading organisms have genetic composition that makes them versatile enough to utilize herbicide as a carbon source. This ability might be total or partial dependent upon the ability of the organism to synthesize and utilize certain enzymes pathways.

A total of thirteen bacterial isolates namely: *Pseudomonas putida*, *Bacillus pumilus, Micrococcus sp., Arthrobacter sp., Lysinibacillus fusiformis, Aeromonas sp., Bacillus cereus, Flavobacterium sp., Acinetobacter rhizoshaerae, Ochrobacterium anthropi, Achrobacterium sp., Bacillus safensis*, and *Achromobacter* sp. were isolated. The study by Munees and Khan (**2011**) revealed that all the rhizobacterial strains generally tolerated the herbicides, glyphosate, up to 3000 µg mL. All bacterial isolates seemed to be evenly distributed in their occurrence in the soils sampled with *Bacillus safensis* and *Lysinibacillus fusiformis* having 85% occurrence. Report of isolation of *Bacillus* spp. in herbicide contaminated soils and their biodegradation abilities are new, however, the available report of isolation of *Bacillus* spp. in herbicide contaminated soils and its ability to degrade herbicide was the one reported by Rohilla and Salar (**2012**).

It is not surprising that all the bacterial isolates upon cultivation in MSA supplemented with 0.1% (v/v) herbicide showed the growth of large member of bacteria. These bacteria are either capable of utilizing the herbicide as carbon source and survive the low toxic conditions of the herbicide (**Munees and Khan, 2011**). This would hold true only if all other dependent variables such as optimal temperature, pH and concentrations of other nutrients are at favourable level. The bacterial isolates showed varying degree of ability to degrade the herbicide. This can be attributed to differences in the expression of necessary enzymes and metabolites that enables them to catalyze utilization of herbicide as carbon source. This conclusion was drawn directly from degree of growth of bacterial isolates on MSM supplemented with 3% of herbicide. *Bacillus* spp. has the highest frequency while *Aeromonas* sp. has the least frequency during the screening period. However, only *Lysinibacillus fusiformis* RB-21, *Bacillus safensis* U17-1, *Bacillus pumilus* GLB197 and *Bacillus cereus* M3 were used in actual biodegradation study in this research as our emphasis was their high frequency during the screening and also *Bacillus* spp. was a lesser known biodegrading organisms in a bid to spark research into the abilities of the organisms so as to broaden the net of research of the organism for the biodegradation of herbicide.

Viable bacterial counts over the 21-day biodegradation period generally followed the typical bacterial growth curve for a batch culture with slight modification comprising of a lag, log/exponential, stationary and death/decline stage. A first glance at values of viable bacterial counts might suggest that a lag phase is missing however, it should be noted that the selected biodegrading organisms were inoculated into a seed inoculums and incubated for 48 hours prior to their inoculation into the bulk MSM supplemented with 2% (v/v) herbicide. Since the isolates were isolated from herbicide contaminated soil, it would be expected that they should not require any acclimatization period, contrastingly, this model would not hold as the soil microenvironment is essentially different from the laboratory artificial medium condition. Hence, an acclimatization period may not only be good but totally essential for the growth of the organisms in the medium.

The log phase of these organisms lasted for 3 days, as between days 3 and 9, the peak of the growth curve was witnessed on the day 9 for *Lysinibacillus fusiformis* RB-21, *Bacillus safensis* U17-1 and *Bacillus pumilus* GLB197 while a steady decline occurs at day 12. This was unlike *Bacillus cereus* M3 whose peak was seen on day 3 and a sharp fall was witnessed on day 6 and subsequently risen on day 9 before a steady decline on day 12. The stationary phases of these biodegrading isolates seem to be embedded in the early stage of the decline phase where the drops in viable counts were steady except for *Bacillus cereus* M3 used.

Measurement of the changes in pH of MSB over a period of biodegradation revealed a steady but continues increase pH value between day 3 to 21 for all bacterial isolates. There was no significant change in pH between day 0 to 3 for *Lysinibacillus fusiformis* RB-21 and *Bacillus safensis* U17-1; a sharp decrease occurs between day 0 and day 3 for *Bacillus cereus* M3 and *Bacillus pumilus* GLB197. The plausible explanation for the fluctuation in pH values is that as microorganisms carry out their metabolic processes, metabolites which alter the pH values are released, which are higher in *Bacillus cereus* M3 and *Bacillus pumilus* GLB197 than *Lysinibacillus fusiformis* RB-21 and *Bacillus safensis* U17-1.

Changes in optical density of each bacterial isolates in MSM largely do not correspond to the observations made in viable population counts. Curve generated for optical density of all isolates between day 0 to day 3 showed a sharp rise over the period of biodegradation day and also a sharp and steady decrease for MSB samples was seen from day 6 to day 12, this further decrease to day 21. Absorbance values peaked for all bacterial isolates on day 3 and subsequently, a steady decline was recorded till day 21.

During the past four decades, a large number of herbicides have been introduced as pre and post-emergent weed killers in many countries of the world. Since herbicides have effectively been used to control weeds in agricultural systems, farmers continue to realize the usefulness of herbicides and larger quantities are applied to the soil. But the fate of these compounds in the soils is becoming increasingly important where they could be leached (in which case groundwater is contaminated) or immobile and persist on the top soil (**Ayansina *et al.*, 2003**). Herbicides could then accumulate to toxic levels in the soil and become harmful to microorganisms, plant, wildlife and man (**Amakiri, 1982**). There is an increasing concern that herbicides not only affect the target organisms (weeds) but also the microbial communities present in soils and these non-target effects may reduce the performance of important soil functions. These critical soil functions include

organic matter degradation, the nitrogen cycle and methane oxidation (**Hutsch, 2001**).

The increased use of herbicides in agricultural soils causes the contamination of the soil with toxic chemicals. When herbicides are applied, the possibilities exist that these chemicals may exert certain effects on non-target organisms, including soil microorganisms (**Simon-Sylvestre and Fournier, 1979**; **Wardle and Parkinson, 1990**). The microbial biomass plays an important role in the soil ecosystem where they fulfill a critical role in nutrient cycling and decomposition (**De-Lorenzo *et al.*, 2001**).

CONCLUSION

Findings from this research showed that most of the indigenous soil microflora has biodegradative potential for glyphosate which its biodegradation product are neither harmful nor toxic, biodegradation is a better and much more attractive method of cleaning up herbicide contaminated soil in contrast to other conventional methods such as incineration and chemical methods which are very expensive and can create environmental problem. Biodegradation of herbicide by soil bacteria and other organisms is eco-friendly, most efficient and economical method of detoxification.

In conclusion, *Lysinibacillus fusiformis* RB-21, *Bacillus safensis* U17-1, *Bacillus cereus* M3 and *Bacillus pumilus* GLB197 can be employed as potentially effective bacterial strains and environmentally safer alternative tools to protect the environment from the pollution of glyphosate containing herbicide.

REFERENCES

Ahemad, M. and Khan, M. S. (2011). Assessment of pesticide-tolerance and functional diversity of bacterial strains isolated from rhizospheres of different crops. *Insight Microbiology,* 1(1): 8-19. https://doi.org/10.5567/IMICRO-IK.2011.8.19

Amakiri, M. A. (1982). Microbial degradation of soil applied herbicides. *Nigeria Journal of Microbiology*, 2: 17-21.

Ayansina, A. D. V., Ogunshe, A. A. O. and Fagade, O. E. (2003). Environment impact assessment and microbiologist: an overview. Process of 11th Annual National Conference of Environment and Behavior Association of Nigeria (EBAN). 26-27.

Baxter, J. and Cummings, S. P. (2008). The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. *Journal of Applied Microbiology*, 104(6): 1605-1616. https://doi.org/10.1111/j.1365-2672.2007.03709.x

Bollag, J. M. and Liu, S. Y. (1990). Biological Transformation Processes of Pesticides, In: Pesticides in the Soil Environment Processes, Impacts and Modelling, Cheng, H.H. (ed.).- Madison, Wis (USA): Soil Science Society of America, 1990.- ISBN 08-911-8791X. p. 169-211.

Brock, T. D., Madigan, M. T., Martinko, J. M. and Parker, J. (1994). *Biology of Microorganisms,* (7th Edition) 192-194.

Buchanan, R. E. and Gibbons, N. E. (1974). *Bergey' Manual of Determinative Bacteriology* (8th Ed.) Williams and Wilkins, Baltimore, 1268 p.

Cullimore, D. R. (2009). *Practical Atlas for Bacterial Identification* (*Sustainable Well Series*) CRC Press 295.

Dick, R. P. (1994). Soil enzyme activities as indicators of soil quality. In: Defining soil quality for sustainable environment. *Soil Science Society of America,* pp: 3-8. https://doi.org/10.2136/sssaspecpub35.c7

Dubey, R. C. and Maheshwari, D. K. (2004). *Practical Microbiology*. S. Chad & Company, New Delhi 162-301.

Ekundayo, F. O. and Osunla, C. A. (2013). Phytase activity of fungi from polluted soils and their ability to degrade Bonny light crude oil. *African Journal of Biotechnology,* 12(36): 5540-5548. https://doi.org/10.5897/AJB2013.12794

Eman, A., Abdel-Megeed, A., Suliman, A., Sadik, M.W., & Sholkamy, E.N. (2013). Biodegradation of Glyphosate by fungal strains isolated from herbicides polluted-soils in Riyadh area. *British Journal of Environmental Science*, 1(1): 7-29.

Fawole, M. O. and Oso, B. A. (2001). *Laboratory Manual of Microbiology* Spectrum Books Limited, Ibadan pp: 71-80.

Frazar, C. (2000). The bioremediation and phytoremediation of pesticide contaminated sites, National Network of Environmental Studies (NNEMS) fellow, Washington DC pp: 3-5.

Garrity, G. M., Bell, J. A. and Lilburn, T. G. (2004). Taxonomic Outline of the Prokaryotes. *Bergey's Manual of Systematic Bacteriology*, 2nd Edition. Release 5.0. Springer-Verlag, New York, May 2004: 1-399. https://doi.org/10.1007/bergeysoutline200405

Hütsch, B. W. (2001). Methane oxidation in non-flooded soils as affected by crop production. *European Journal of Agronomy*, 14(4): 237-260. https://doi.org/10.1016/S1161-0301(01)00110-1

Kinney, C.A., Mosier, A.R., Ferrer, I., Furlong, E.T. and Mandernack, K.W. (2004). Effects of the herbicides prosulfuron and metolachlor on fluxes of CO_2, N_2O, and CH_4 in a fertilized Colorado grassland soil. *Journal of Geophysical Research D: Atmospheres,* 109(5):12p https://doi.org/10.1029/2003JD003656

Okerentugba, P. O. and Ezeronye, O. U. (2003). Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluent in Nigeria. *African Journal of Biotechnology,* 2(9): 288-292. https://doi.org/10.5897/AJB2003.000-1058

Olutiola, P. O., Famurewa, O. and Sonntag, H. G. (1991). Types of Media an Introduction to General Microbiology: A Practical Approach. 1st edition, Heidelberger Verlagsanstalt Druckerei GmbH, Heidelberg, Germany, pp: 48-50.

Pramer, D. E. and Schmidt, E. L. (1964). *Experimental Soil Microbiology*. Burges Publishing Company Minneapolis 15. Minnesota, USA. Sons Ltd. Beecles and London, 31-32.

Prasad, S., Srivastava, S., Singh, M. and Shukla, Y. (2009). Clastogenic Effects of Glyphosate in Bone Marrow Cells of Swiss Albino Mice. *Journal of Toxicology,* 10: 115-161. https://doi.org/10.1155/2009/308985

Relyea, R. A. (2005). The Impact of Insecticides and Herbicides on the Biodiversity and Productivity of Aquatic Communities. *Ecological Applications,* 15(2): 618-627. https://doi.org/10.1890/03-5342

Rohilla, S. K. and Salar, R. K. (2012). Isolation and characterization of various fungal strains from agricultural soil contaminated with pesticides. *Research Journal of Recent Sciences,* 1 (ISC-2011): 297-303.

Saeki, M. and Toyota, K. (2004). Effect of bensulfuron-methyl (a sulfonylurea herbicide) on the soil bacterial community of a paddy soil microcosm. *Biology and fertility of soils,* 40(2): 110-118. http://dx.doi.org/10.1007/s00374-004-0747-1

Salbego, J., Pretto, A., Gioda, C. R., Menezes, C. C., Lazzari, R. N., Joao, B. and Loro, V. L. (2010). Herbicide Formulation with Glyphosate affects Growth, Acetycholinesterase Activity and Metabolic and Hematological Parameters in Piava (*Leporinus obtusidens*). *Archives of Environmental Contamination and Toxicology,* 58(3): 740-745. https://doi.org/10.1007/s00244-009-9464-y

Sannino, F. and Gianfreda, L. (2001). Pesticide influence on soil enzymatic activities. *Chemosphere,* 45(4-5): 417-425. https://doi.org/10.1016/s0045-6535(01)00045-5

Sebiomo, A., Ogundero, V. W. and Bankole, S. A. (2011). Effect of four herbicides on microbial population, soil organic matter and dehydrogenase activity. *African Journal of Biotechnology,* 10(5): 770-778. https://doi.org/10.4314/AJB.V10I5

Solomon, K. and Thompson, D. (2003). Ecological Risk Assessment for Aquatic Organisms from Over-Water Uses of Glyphosate. *Journal of Toxicology and Environmental Health*, Part B 6(3): 289-324. https://doi.org/10.1080/10937400306468

Stephen, O. D. and Stephen, B. P. (2008). Glyphosate: a once-in-a-century herbicide: Mini-review. *Pest Management Science,* 64(4): 319-325. https://doi.org/10.1002/ps.1518

Sviridov, A. V., Shushkova, T. V., Zelenkova, N. F., Vinokurova, N. G., Morgunov, I. G., Ermakova, I. T. and Leontievsky, A. A. (2012). Distribution of glyphosate and methylphosphonate catabolism systems in soil bacteria *Ochrobactrum anthropi* and *Achromobacter* sp. *Applied Microbiology and Biotechnology,* 93(2): 787- 796. https://doi.org/10.1007/s00253-011-3485-y

Wardle, D. A. and Parkinson, D. (1990). Effects of three herbicides on soil microbial biomass and activity. *Plant Soil,* 122: 21-28. https://doi.org/10.1007/BF02851906

Xiao, P., Mori, T., Kamei, I. and Kondo, R. (2011). Metabolism of Organochlorine Pesticide Heptachlor and its Metabolite Heptachlor Epoxide by White rot Fungi, Belonging to Genus *Phlebia*. *FEMS Microbiology Letters,* 314(2): 140-146. https://doi.org/10.1111/j.1574-6968.2010.02152.x