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RESEARCH ARTICLE

EFFECT OF LONG TERM EXPOSURE TO GLYPHOSATE HERBICIDE ON SOIL MICROBIAL POPULATION DYNAMICS.

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Abstract

Glycophosate herbicide is one of the most widely used herbicides by farmers because of its efficacy. However, little is known about its possible long term effects on important soil microflora. To investigate this, mesocosm study involving four cell, Cell I, II, III, and IV contaminated with the herbicide at concentrations of 5, 20, 50 and 0 % v/v respectively were studied between October, 2016 to March, 2017. Soil samples analysed weekly from respective cells to determine the effect of herbicide on overall heterotrophic bacterial and fungal populations, and specifically on *Nitosomonas*, *Nitrobacter* and actinomycetes populations, qualitatively and quantitatively with time. The results showed that total heterotrophic bacteria counts (THBC) and actinomycetes counts decreased with time and concentrations for the first 28 day as compared to the control before attaining steady increases. However, beyond day 90, THBC and actinomycetes counts in control cell (Cell IV) were lower than all other cells with the exception of Cell III ($P < 0.5$). The total fungal counts (TFC) were not significantly affected except for Cell I that had higher TFC than other cells at day 180 ($P < 0.5$). Populations of *Nitrosomonas* and *Nitrobacter* witnessed a progressive decrease with time and concentrations as compared to uncontaminated soil ($P < 0.5$). The following genera of bacteria, *Pseudomonas*, *Bacillus*, *Acinectobacter*, *Micrococcus*, *Alcaligens*, *Achromobacter* and fungi, *Rhizopus*, *Trichoderma*, *Penicillium* and *Aspergillus* were isolated. *Actinomyces* and *Nocardia* were the actinomycetes isolated. Though bacteria and fungi in soil generally recovered from the effect of glyphosate application in the long term following initial population decline except at the highest concentration of 50 % v/v, *Nitrosomonas* and *Nitobacter* (nitrogen fixers) specifically never recovered. This portend serious problem for nitrogen fixation in soil.

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Introduction:-

Right from inception of agriculture, there has been an overly need to control weeds. Importantly, in third world countries like ours, much energy and time is spent in weed control as this, is usually done mechanically, by the use of farm tools or implements such as cutlass, hoes and harrows powered by animals. This method of weed removal, have greatly hindered the extent to which arable farms can be done in terms of size hence further, affecting the overall food supply and in fact, foreign exchange in these developing nations (Nigeria a typical example). This inherent problem of shortage in food supply has increasingly necessitated the use of herbicides and other agrochemicals not just in tropical countries but worldwide.

In arable farming, herbicides with selected spectrum of activity are used to selectively kill the weeds while not damaging the crops. At the moment the use of glyphosate, one of many other herbicides is on the increase especially in the Niger Delta region of Nigeria (Sebiomo *et al*., 2011).This is probably due to the supposed efficacy of the herbicide.

Glyphosate is an amino acid inhibitor which kills plants by preventing the synthesis of certain amino acids like valine, leucine and isoleucine which are needed for the synthesis of deoxyribonucleic acid as well as cell growth. Plants treated with amino acidn inhibitor dye slowly; consequently increasing the time for re-emergence of the weed and thereby reducing overall weed/crop competition. This could be accountable for the wide acceptance of glyphosphate. Although, the use of glyphosate has gained popularity in the agricultural sector, its effect on soil microbial diversity have not been quite elucidated especially in the rainforest region of Nigeria (Atlas *et al*., 1991; Bromilow *et al*., 1996 and Johnson *et al*., 2001).

Microorganisms play important role in terrestrial ecosystems especially in balancing the various biogeochemical cycles, improvement of soil fertility and the degradation of various organic as well as xenobiotic compounds hence it is necessary to determine the possible effects the prolonged application of this widely accepted herbicide -glyphosate could have on soil microbial populations with the ultimate objective or goal of establishing bio-indicators of ecosystem perturbations caused by glyphosate herbicide.

Materials And Methods:-

Selection of test herbicide (Glyphosate):-

Glyphosate herbicide was preferentially selected for this study among the sampled herbicides (Glyphosate, Dichlorvous, Paraquat and 'Unkown'). This choice was based on the results of preliminary investigation from one hundred farmers (cassava, oil palm, leafy vegetables, tomatoes and maize farm owners) interviewed across the twenty five local government area of Delta State, Nigeria, on the most commonly applied herbicides (Table 1). Glyphosate used in this study was purchased from the open market in Abraka, Ethiope-East Local Government Area of Delta State.

Preparation of mesocosms:-

Mesocosm study was carried out for a six month period (October, 2016 to March, 2017). Four wooden cells of 1m x 1m x 1m were constructed on an open farm land in Abraka, Ethiope-East Local Government Area of Delta State. These four cells were labelled Cell I, Cell II, Cell III and Cell IV. The soil within each cell was then properly tilled and a ridge in each cell of about 40 cm high was made. Top soil sample (0-3 cm depth) was taken to the laboratory for immediate analysis of physico-chemical properties of the soil.

Baseline physicochemical properties of soil:-

Soil used for this study was analysed for nitrogen, total organic carbon (TOC), phosphorus, pH, porosity and texture to determine baseline properties using the methods of micro-Kjeldahl procedure (Hesse, 1979), Black (1965), Bray and Kurtz (1971), Black (1965), Ezzati *et al*. (2012) and hydrometer method as outlined by Aliyu and Oyeyiola (2011) respectively.

Preparation of herbicide concentrations and application:-

Three concentrations [5, 20 and 50 (% v/v)] of the herbicide (glyphosate) were prepared using sterile deionized water. One hundred millilitres (100 ml) of each herbicide concentrations was applied to soil by spraying (mimicking the usual field application procedure). Cells I, II and III received 5, 20 and 50 (% v/v) herbicide respectively while Cell IV served as control (receiving 100ml of sterile deionized water only).

Microbiological evaluation of soil samples:-

Triplicate soil samples collected on each analysis day (day 0, 7, 14, 21, 28, 60, 90, 120, 150 and 180 of experiment) were evaluated microbiologically (within 24 hours) to determine total bacterial and fungal load as well the various bacterial and fungal genera present.

Determination of bacterial and fungal load involved diluting soil specimen using the ten-fold serial dilution technique and inoculating 0.1ml of various dilution factors ranging from 10^{-3} to 10^{-6} on Standard Plate Count agar (for total heterotrophic bacteria), Yeast Extract Mannitol agar (for *Rhizobium* count), Winogradsky phase I and II agar (for *Nitrosomonas* and *Nitrobacter* counts respectively), Actinomycetes agar for the enumeration of

Actinomycetes and malt extract agar for the quantification of fungi. All plates were incubated at ambient (28 ± 2 ^{0}C) temperature for 18-72h with the exceptions of Winogradsky phase I and II agar plates which were incubated for 7-14 days. At the end of incubation period, distinct colonies were counted. It is important to state that the appropriate dilution factor for each microbial group was noted at day 0 and used for subsequent analysis.

Identification of Isolates:-

Identification of bacterial isolates was based on observation of cellular and colonial morphologies in addition to biochemical characterization according to guidelines in Bergey's manual of determinative bacteriology. However, fungal identification was performed by observation of colonial morphology as well as mcroscopic considerations of conidia, sporangia and mycelia arrangements (Barnett and Hunter, 1972).

Results:-

Prevalence of herbicide used by various farmers in Delta State:-

At the end of oral interviews conducted among farmers across Delta State, Nigeria, it was observed that glyphosphate herbicide had highest patronage/use as depicted in Table 1 below.

Physico-chemical properties of soil:-

The result of the Physico-chemical properties of soil sample used in this study is as presented in table 2. Results indicated that soil is slightly acid and may be described texturally as loamy sand.

Effect of herbicide on total heterotrophic bacterial and fungal counts:-

Results of the effect of glyphosphate herbicide on total heterotrophic bacteria count is as represented in Figure 1. It was noticed that the total heterotrophic bacteria count reduced significantly ($P < 0.05$), with both increasing herbicide concentration and period of exposure up until the first 28 days. Thereafter, at all concentrations of exposure, there were increases in the number of heterotrophic bacteria quantified. Consistently, the total heterotrophic bacteria counts in control mesocosm were significantly higher than in cells that received various herbicide concentrations. However, beyond day 90, THBC in control cell were lower than in all other cells with the exception of Cell III which received the highest concentration of herbicide (50% v/v). The results of the total fungal count obtained are as shown in Figure 2. Heterotrophic bacterial isolates obtained belonged to six genera which include; *Pseudomonas, Bacillus, Acinectobacter, Micrococcus, Alcaligens* and *Achromobacter*. While the following genera of fungi, *Rhizopus, Trichoderma, Penicillium* and *Aspergillus* were isolated from contaminated soil. (Table 3).

Generally, the total fungi counts obtained on each day of analysis were lower than thecounts of total heterotrophic bacteria. While some bacterial species were never isolated within the period of study, all the fugal isolates persisted throughout the study period.

The results of total *Nitrosomonas* and *Nitrobacter* counts are as presented in Figure 3 and 4 respectively. Both organisms were considerably, sensitive to all concentrations of glyphosphate used, such that beyond day 7, *Nitrosomonas*sp and *Nitrobacter*sp were no longer recovered from cells that received 20 and 50 (%v/v) herbicide concentration. Cell I into which 5 %v/v herbicide concentration was introduced demonstrated a consistent decline in the population of *Nitrosomonas* sp and *Nitrobacter* sp up until day 60 after which these organisms were no longer isolated.

The glyphosate herbicide caused the actinomycetes population in each cell to dwindle for the first 28 days with the exception Cell IV (control) whose actinomycetes population remained relatively constant throughout the study period. It was noticed that a re-proliferation of actinomycetes in all cells occurred beyond day 28, the genera of Actinomycetes isolated were *Actinomyces* and *Nocardia*. Also, there was significant difference in actinomycetes population obtained from various cell. The result of total Actinomycetes count is as represented in Figure 5.

Table 1:- Common herbicides and their frequency of usage by farmers in Delta state, Nigeria.

Herbicide	Frequency of Usage (%)
Glyphosate	63
Dichlorvous	17
Paraquat	10

*Unkown	10

*= Persons who couldn't give the name or provide cans of herbicides they use.

Table 2:-Physico-chemical properties of soil

Parameter	Values
TOC (%)	0.02
Nitrogen (%)	0.15
Phosphorus (mg/kg)	41.70
Porosity (%)	61.0
pH	6.60
Sand	88.0
Silt	5.20
Clay	6.80

Table 3:- Fungal and bacterial genera isolated from soil

Fungi	Hetrotrophic Bacteria	Nitrogen Fixers	Actinomycetes
Aspergillus	*Pseudomonas*	*Nitosomonas*	*Actinomyces*
Penicillium	*Bacillus*	*Nitrobacter*	*Nocardia*
Rhizopus	*Acinectobacter*		
Trichoderma	*Micrococcus*		
	Alcaligens		
	Achromobacter		

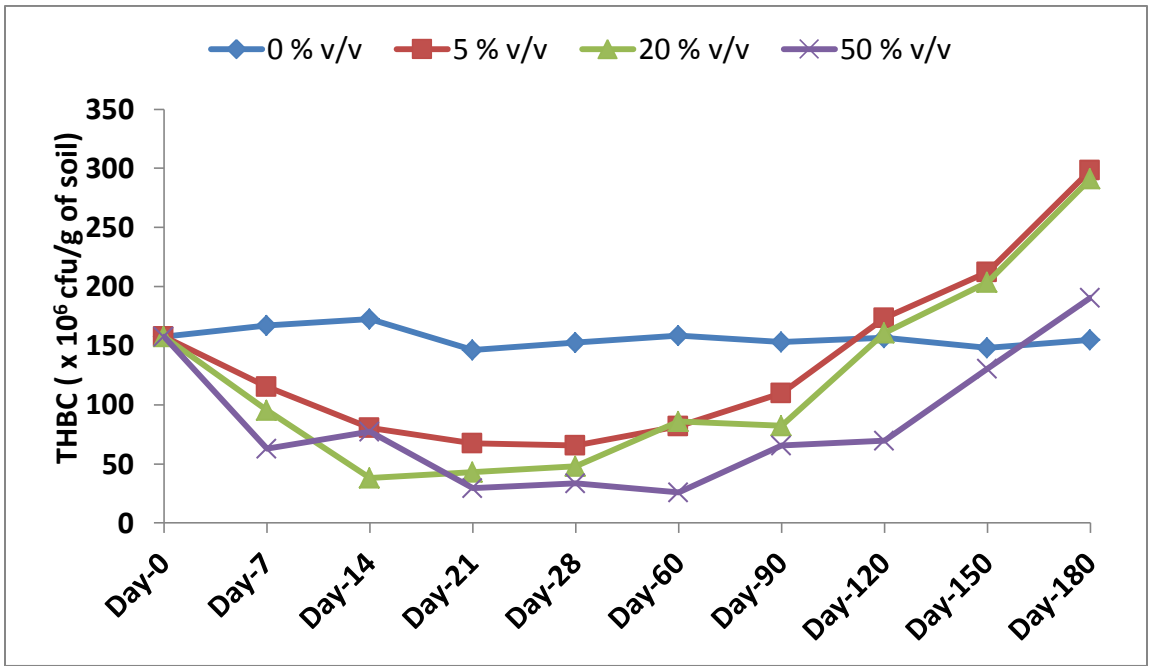

Figure 1:- Effect of glyphosate herbicide on total heterotrophic bacterial counts (THBC) in soil.

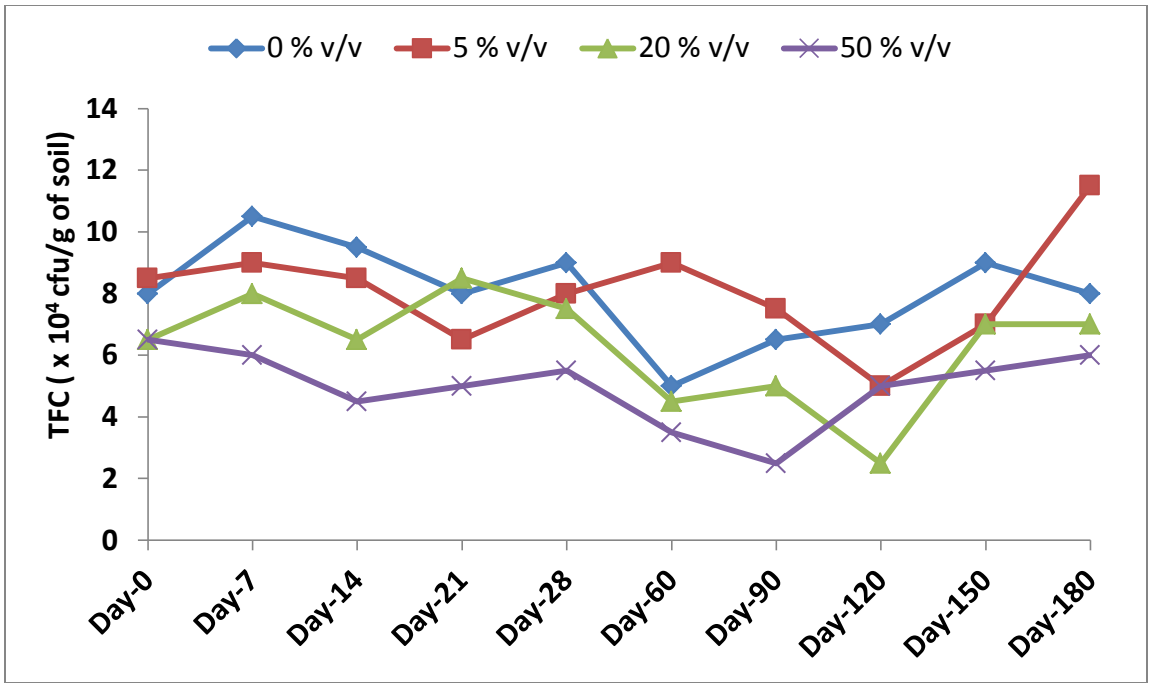

Figure 2:- Effect of glyphosate herbicide on total fungal counts (TFC) in soil

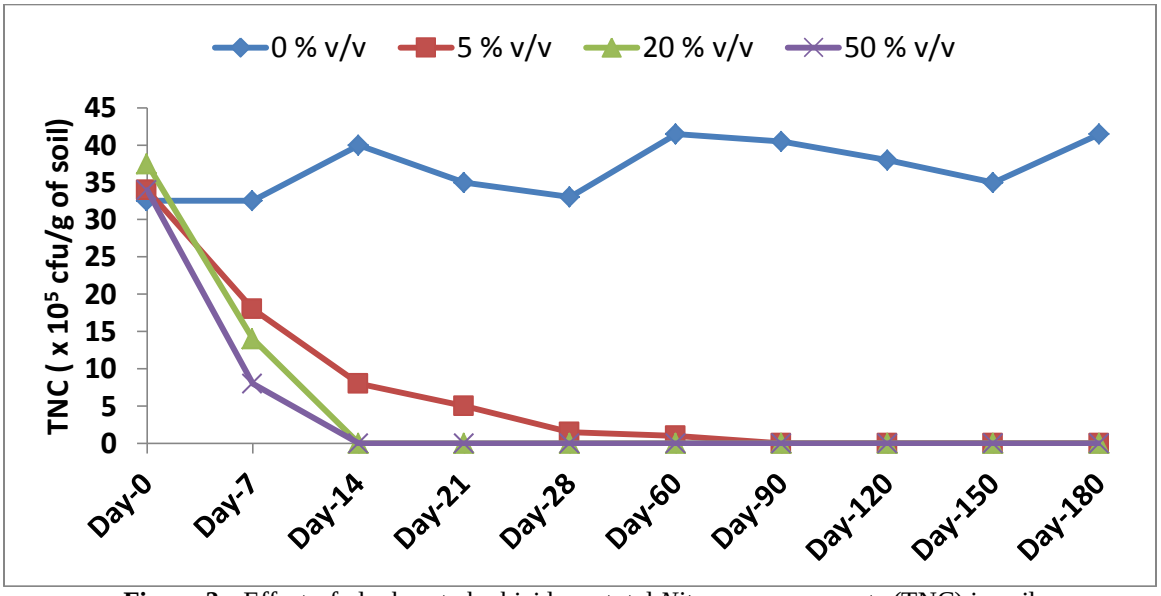

Figure 3:- Effect of glyphosate herbicide on total *Nitrosomonas* counts (TNC) in soil

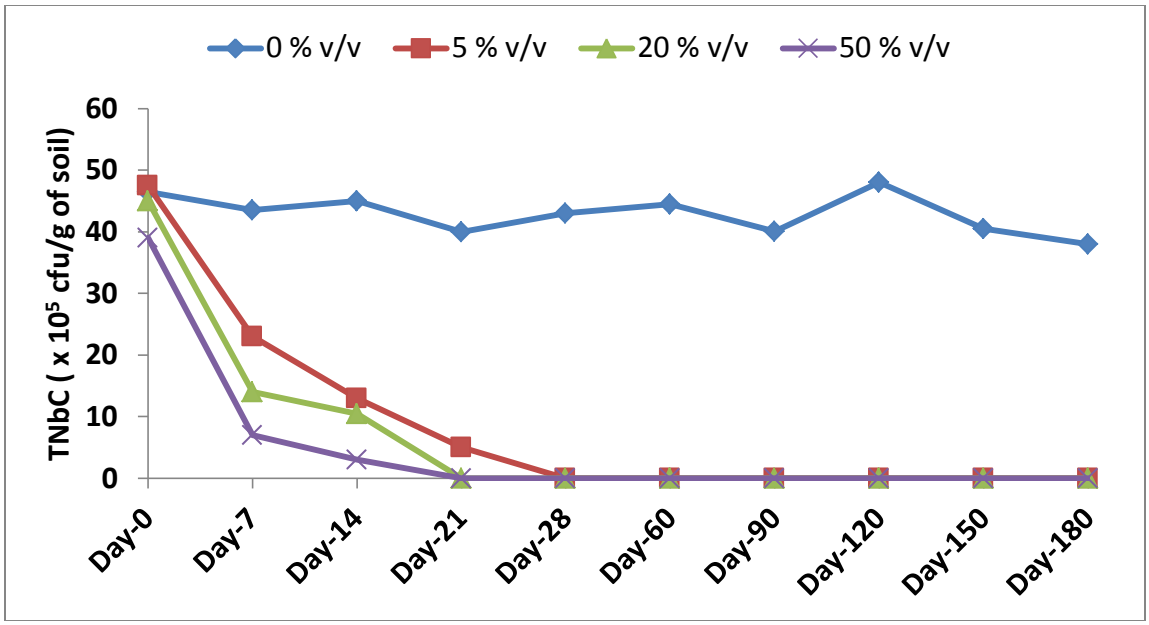

Figure 4:- Effect of glyphosate herbicide on total *Nitrobacter* counts (TNbC) in soil

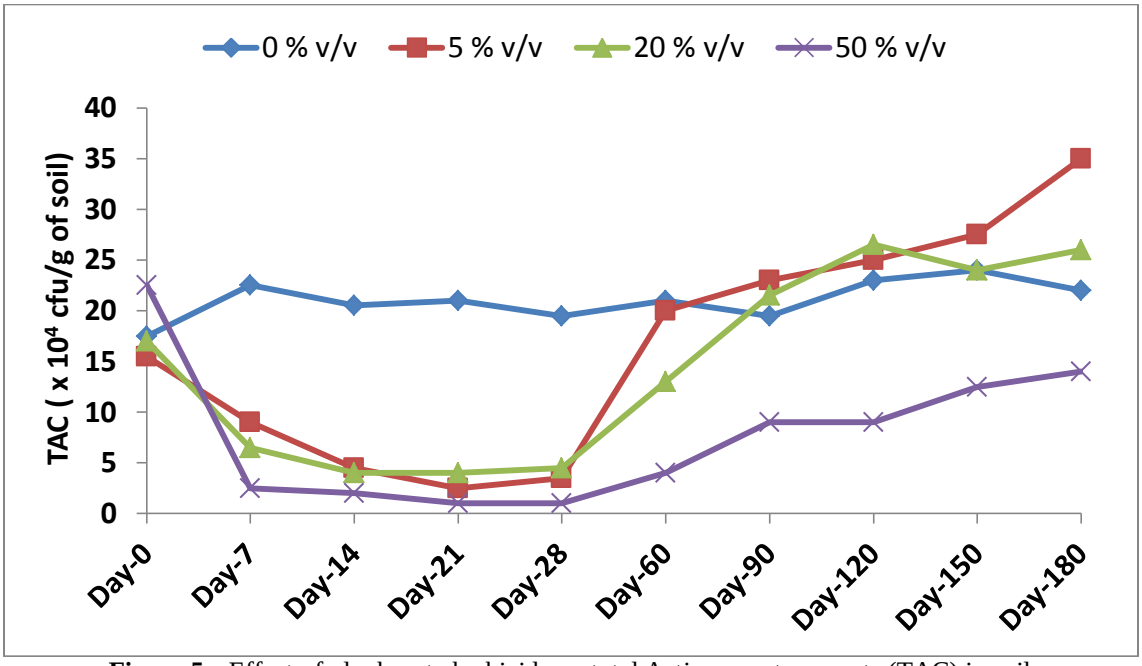

Figure 5:- Effect of glyphosate herbicide on total Actinomycetes counts (TAC) in soil

Discussion:-

The initial drop in the total heterotrophic bacteria count (first 28 days) observed in this study was most likely as a result shock response of the heterotrophic bacteria community to the toxicity of glyphosate. Several reports including Rath *et al*., 1988 ; Johnson *et al*., 2001 and Zhang *et al*., 2010, have presented similar results indicating the capability of various pesticides to suppress the growth of certain bacteria. In agreement with results of the above listed authors, this study revealed selective deleterious effect on Gram negative bacteria. The disappearance of certain bacterial groups (majorly gram negative) in this study is suggestive of the fact that the gram positive bacteria were more of strategist, could adapt and perhaps could resist/utilize the chemical and hence, persisted and dominated with time.

At the juncture (that is beyond day 28) where elevation were noticed in total heterotrophic bacteria count, one may attribute it to either one or more of the following: the recovery of at least some of the previously suppressed bacteria; the proliferation of glyphosate utilizing bacteria; the increases in the population of bacteria that had the ability of breaking down the herbicide. Also, possible reductions in available herbicide concentrations in the soils due to natural attenuation, adsorption to soil particles or even leaching could also account for the re-emergence of heterotrophic bacteria population observed in the later days of analysis. Suffice to state that glyphosate have a high binding capacity to soil and a relatively short half life of 47 days. Beyond day 90, efficient loss of the herbicide was evident in cells I and II and might be responsible for the total heterotrophic bacteria counts becoming significantly higher than in control cell. Sebiomo*etal*. (2011) also corroborates the fact that herbicides significantly reduces bacterial populations in soils but that herbicide treated soils could recover after six weeks of treatment. In same vein, Zhang *et al*., 2008, reported the stimulatory effect of cypermethrin on total viable bacterial population.

The persistence of the few fungi isolates that occurred in the various cells, is an indication of the resilence and the resistance of these group of organisms to the herbicide. This may be due to their genetic makeup or perhaps to the possession of certain enzymes capable of breaking down glyphosate. It is likely that the initial activities of fungi in this study, created the room for the observed re-emergence of certain bacterial isolates. Furthermore, Sebiomo *et al*., 2011 observed fluctuations in fungal load in herbicide treated soils while noting that actinomyces could interact favourably with glyphosate. Results obtained in this study are at par with this. On another hand, the drastic reduction observed in the population of Nitrosomonas and Nitrobacter at all tested concentrations, is an indication of cells' death or probably cells undergoing the viable but non-culturablephenonmenon. Similarly, Moriora *et al*., 2015 reported that cypermenthrin adversely decreased populations of nitrogen fixers (such as *Azotobacter* spp, *Clostridium* spp), ammonifying, nitrifying and denitrifying bacteria. Various authors (including, Akponah *et al*., 2014, Odokuma and Akponah, 2008) have shown that *Nitrosomonas* and *Nitrobacter* are highly sensitive to a wide range of Xenobiotics. Infact, the behaviour of these two isolates in terms of responses to toxic chemicals have presented them as excellent tools in ecotoxicity assessment. Again, these isolates have indicated the toxic potential of glyphosate even at very low concentrations.

Conclusively, the study re-iterates the eco-toxicological importance of the herbicide glyphosate establishing its possible effects in the reduction of bacterial population that would otherwise play significant roles in the biogeochemical cycles, biodegradation, carbon mineralization, nitrification process and ultimate improvement of soil health and fertility. Its prolonged use may considerably alter the 'eco balance'. Although, the diversity in microbial community in rainforest soils receiving glyphosate had been shown not to be robust, the study suggest strongly that there are a few organisms likely possessing the capability of withstanding this toxic chemical; it is therefore recommended that these organisms should be selected for possible strain improvement and used as tools for the subsequent rapid clean-up or removal of this inimical substance from soils where they are frequently utilized especially in the rainforest region of Nigeria.

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