Available online www.jsaer.com

***Journal of Scientific and Engineering Research*, 2015, 2(4):55-63**

Research Article

ISSN: 2394-2630
CODEN(USA): JSERBR

Effects of Pesticides on the Micro-Flora of Loamy Soil obtained from Biological Garden, Federal University of Technology, Minna, Nigeria

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Abstract This study was conducted to determine the effects of pesticide application on soil microbial spectrum using a herbicide (Glyphosate; the active ingredient in Roundup) and an insecticide (Chlorpyrifos). The control soil samples had no pesticide application. Soil samples taken at the depth of 0-20cm from the Biological Garden was taken to the laboratory. The soil was sieved through a 2mm mesh to remove stones and plant debris. One kilogram of the soil samples was weighed and transferred into different containers into which 150ml of the dissolved pesticides were applied. The numbers of colony forming units (CFU) of respective groups of microorganisms were determined on Nutrient Agar and Sabouraud Dextrose Agar media by pour plate method. After 1, 7, 14 and 21 days of experiment, the number of bacteria and fungi were estimated. At the end of the study, results revealed that pesticide applications caused reduction in microbial population present in the soil when compared with the control. Also, despite the reductions, bacterial species like *Bacillus subtilis* developed tolerance possibly due to its characteristic spore formation, others like *Pseudomonas aeruginosa* disappeared immediately after pesticide treatment only to reappear and start multiplying after some days. This may be due, in part, to evolution and development of metabolic mechanisms to use the pesticides as a carbon and energy source. Using a paired t-Test, calculated value of 14.9 for Glyphosate and 24.1 for Chlorpyrifos is greater than t-tabulate value at 0.05 confidence level, indicating that there is a great difference between microbial population in pesticide treated soil and non-treated soil confirming the effects of microbial population reduction and possible extinction due to pesticide application on the soil.

Keywords Pesticides, Microbial flora, Loamy soil, Biological garden, CFU

Introduction

Pesticides are defined under the United State Federal Environmental Pesticide Control Act (FEPCA) as “any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (insects, rodents, nematodes, fungus, weeds and other forms of terrestrial or aquatic plant or animal like bacteria or other microorganisms)” or “any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant”. Pesticides are directly toxic to pests having indirect effects to soil invertebrates [1]. It was observed that physicochemical properties of the soil, nature of substrates and environmental degradation determine the persistence of pesticides in nature. Excessive persistent and biological active residues endanger non-target organisms, prove hazardous and make the pest control operational uneconomical [1-2].

In recent time there has been a steady increase in the number and amount of pesticide residues in our food and soil. While pesticides serve useful purposes, concern has been expressed regarding their possible effects on environment. Effects of pesticides on living organisms in the soil are as follows:

- They may be directly toxic to animal in soil
- They may affect the soil organisms genetically to produce population resistant to the pesticides
- They may have sub-lethal effects that result in alterations in behavior or changes in metabolic or reproductive activity.
- They may be taken into bodies of soil flora or fauna and passes on to the other organisms.

Based on these effects, [4] observed that, the problem of pests in agricultural practice cannot be solved thorough the continued and exclusive application of broad-spectrum pesticides. Implementation of Integrated Pest Management (IPM) programmes that institute ecologically sound and multi-component suppression on the pest population is the obvious solution.

The soil microorganisms like bacteria, fungi, algae and nematodes play important role in soil nutrition through their role in decay of plant and other organic matter in soil. Anything that disrupts their activity could be expected to affect the nutritional quality of soils and would thus have serious consequences. Also, microorganisms that live in soil can be killed not only by chemicals applied directly to the soil, but also by those that reach the soil in drift from aerial sprays or washed off foliage, which in turn affect the breakdown of some kinds of dead leaf material into its organic and inorganic constituents and in the incorporation of these material into the soil structure [1].

Continuous use of pesticides requires constant monitoring with respect to their persistence in soil and plants and effects on soil organisms in terms of ill effects and toxic residue. Soil microorganisms have a great contribution towards soil fertility. Any adverse impact of chemical on soil characteristics and microorganism may lead to ultimate loss of soil fertility.

Although pesticides have benefits, some also have drawbacks, such as potential toxicity to humans and other desired species. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 most dangerous and persistent organic chemicals are organochlorine pesticides [5].

Glyphosate

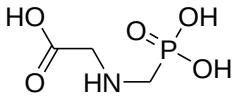

Figure 1: Chemical Structure of Glyphosate

IUPAC Name: N-(phosphonomethyl)glycine

Properties

Molecular formula: $C_3H_8NO_5P$

Molar mass: 169.07 $gmol^{-1}$

Appearance: White Crystalline Powder

Density: 1.704 (20 °C)

Melting Point: 184.5 °C

Boiling Point: Decomposes at 187 °C

Solubility in Water: 1.01 g/100ml (20 °C) [6]

Chlorpyrifos

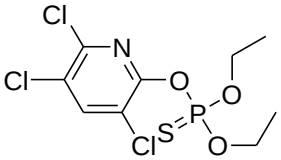

Figure 2: Chemical Structure of Chlorpyrifos

IUPAC Name: *o,o*-Diethyl-*o*-3,5,6-trichloropyridin-2-yl phosphorothioate

Other Names: Brodan, Detmol UA, Dowco 179, Dursban, Empire, Eradex, Lorsban, Paqeant, Piridane, Scout, Stipend and Tricel.

Properties:

Molecular formula $C_9H_{11}C_{l3}NO_3PS$

Molar mass 350.59 g/mol

Appearance colourless crystals [7]

Density 1.398 g/cm 3 (43.5 °C)
Melting point 42 °C
Solubility in water 2 mg/L (25 °C) [8]

Materials and Methods

Soil Sample Collection

Top soil sample (0-20 cm deep) was collected from two locations at the Biological Garden within FUT MinnaBosso Campus, Niger State and was taken to the Microbiology Laboratory for analysis. The soil samples was first sieved through a 2.00 mm width mesh to remove stones and plant debris and then using a weighing balance, one kilogram of the soil sample was transferred into four different containers.

Pesticides and application

Two pesticides, Glyphosate and Chlorpyrifoswere used for this research. They were purchased from an agro-allied market in Minna, Nigeria.Using a sprayer, two containers of one kilogram soil sample was treated with one and half times (x1.5ai) of recommended doses of each pesticide (glyphosate and chlorpyrifos). One kilogram of the soil samples in two containers was separately treated with x1.5 doses (ai) of each of the pesticides, while the third and fourth soil sample from different locations was treated with distilled water to serve as control. The moisture content of soil was maintained throughout the study by periodic addition of distilled water, establishing 45 % to 50 % of the soil maximum water-holding capacity. To avoid evaporation of water from the soil and photodegradation of pesticides, the containers were covered with polypropylene sheets and incubated in the dark. All soil samples were incubated at 20±2 ^{o}C for 21 days.

Analytical procedure

Microbiological analysis of the soil samples were made as per the following procedures:
For the enumeration of the micro-fungal and bacterial population, Dilution Plate Count Technique (DPCT) was followed.
The primary suspension of the soil samples was prepared from 1 gram of each soil which was diluted up to 10^{-9} times using sterile water as diluting fluid.

- For microfungal population, 1ml of aliquots from 10^{-4} diluted suspension was transferred to Petri dishes using sterile syringes and to it 25 ml of Sabouraud Dextrose Agar medium using pour plate method. The petri dishes were incubated at room temperature for the development of fungal colonies.
- For the enumeration of bacterial population, 1ml of aliquots from 10^{-6} diluted suspension was inoculatedinto Petri disheswith molten Nutrient Agar medium. The Petri dishes was incubated at 37 ^{o}C for 24 hours.

Isolation of Bacterial Colonies

After 24 hours of incubation, the total bacterial colonies from the two control soil samples were counted and recorded. Individual distinct colonies with similar colonial morphology were also counted separately and recorded. The total bacterial colonies from the glyphosate and chlorpyrifos treated soil samples were also counted. The distinct colonies observed on the control samples were also observed on the pesticides treated soil samples and their numbers were recorded. Soil samples from the pesticide treated soils were analyzed every week for a period of four weeks. The pure colonies were later transferred to agar slants for storage and further identification.

Biochemical Tests for Identification

Bacteria isolation and characterization were done using Gram's Staining, Endospore Staining, Oxidase Test, Catalase Test, Citrate Utilization Test, Methyl Red Test and Sugar Fermentation Test.

Isolation and Identification of Fungal Colonies

After 48 hours of incubation, the total fungal colonies from the control soil sample were counted and recorded. Individual distinct colonies with similar colonial morphology were also counted separately and recorded. The total fungal colonies from the glyphosate and chlorpyrifos treated soil samples were also counted at 7 days interval. The distinct colonies observed on the control samples were also observed on the pesticides treated soil samples and their numbers were recorded. The fungal colonies were identified by their cultural morphology and by microscopic examination.

Figure 3: The two pesticides used in this experiment- Glyphosate &Chlorpyrifos

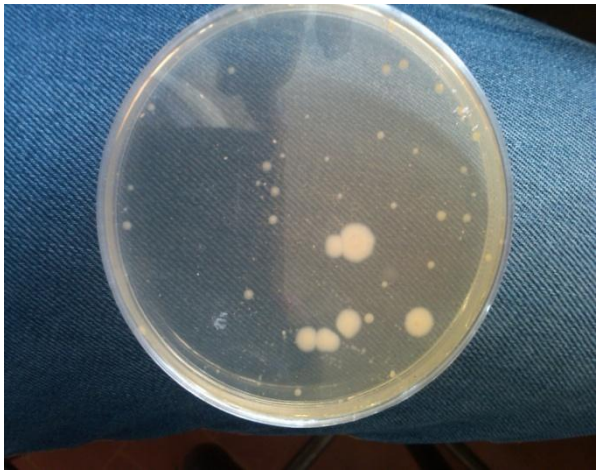

Figure 4: Bacterial colonies growing on the petri dish

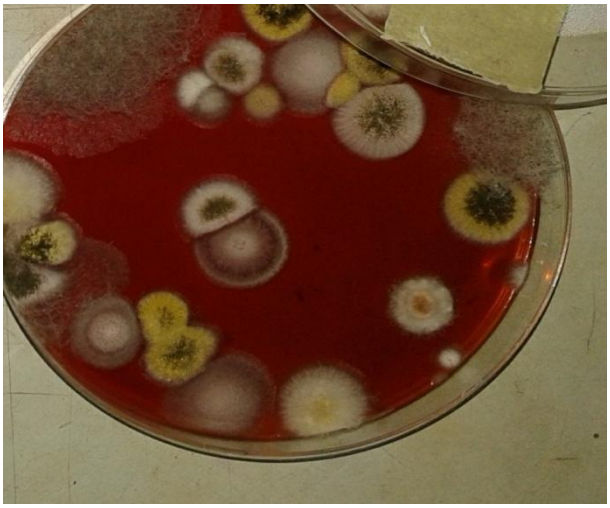

Figure 5: Fungal colonies growing on the petri dish after 48 hours of incubation

Table 1: Biochemical Reactions of the Bacterial Isolates

Isolates	Gram Reaction	Catalase	Endospore	Oxidase	Methyl Red	Citrate	Sugar Fermentation Test				Suspected Organism
							Sucrose	Lactose	Glucose	Gas	
Isolate 1	+ve rods	+	+	−	−	−	+	−	+	+	*Bacillus subtilis*
Isolate 2	-ve rods	+	−	+	−	+	−	−	−	−	*Pseudomonas aeruginosa*
Isolate 3	-ve rods	+	−	−	+	−	+	+	+	+	*Escherichia coli*
Isolate 4	-ve rods	+	−	−	+	+	+	−	+	+	*Proteus* spp

Keys: + Reactive,− Non-Reactive

Table 1 shows the biochemical characterization of bacteria isolated from the control soil sample. The bacteria colonies were characterized by colonial morphology and also, series of biochemical tests were carried out to identify the bacterial isolates. The major bacteria colonies found in the soil were Gram positive and Gram negative rods which includes *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus* spp.

Table 2: Characterization of Fungal Colonies

Morphological Characterisitic	Microscopic Examination	Suspected Organism
Flat velvety filamentous woolly texture with green colony	Septatehyphe with simple conidiophore and round conidia	*Penicillium*spp
White and wooly aerial growth that darkens as it sporulates	Non-septate hyphae with straight sporangiophore with many spherical spores	*Mucor*spp
Velvety filamentous white/yellow growth that produces black powdery spores	Long septate hyphae with conidiophore bearing brown spores and phialides at its apex	*Aspergillus*spp

Table 2 shows the major fungal colonies observed on the agar plate. The colonies were characterized based on their colonial morphology and microscopic examination. *Penicillium*spp, *Mucor*sppand *Aspergillus*spp were the major fungal species identified and quantified.

Table 3: Fungi isolates from soil not treated with pesticide

S/N	Fungi	Colony Count A	Colony Count B	Mean
1.	*Penicillium*spp	6	7	6.5
2.	*Mucor*spp	7	8	7.5
3.	*Aspergillus*spp	22	24	23
4.	Others	10	8	9
	Total	45	47	
	Mean	11.25	11.75	

Table 3 shows the number of fungi isolates in the soil samples not treated with Herbicide Glyphosate and Insecticide Chlorpyrifos. From table 3, *Aspergillus*spp with its characteristic black spores had the highest number with an average 23×10^4cfu/g. This was followed by *Mucor*sppand *Penicillium*sppwhich occurred in modest numbers.

Table 4: Fungi isolates from soil treated with glyphosate

S/N	Fungi	Day 1	Day 7	Day 14	Day 21
1.	*Penicillium*spp	2	2	1	4
2.	*Mucor*spp	6	4	3	5
3.	*Aspergillus*spp	17	15	12	23
4.	Others	8	5	3	5
	Total	33	26	19	37
	Mean	8.25	6.5	4.75	9.25

Table 4 shows the number of fungi isolated from soil samples treated with glyphosate. The colony counted on Day 1 was 33×10^4cfu/g which is a lower figure than that observed in the control sample. The colony count further reduced after Day 7 and Day 14 of sampling having 26×10^4cfu/g and 19×10^4cfu/g respectively. Day 21 showed an increase in the colony count with 37×10^4cfu/g which was higher than the colony count observed on the first sampling day.

Table 5: Fungi isolates from soil treated with chlorpyrifos

S/N	Fungi	Day 1	Day 7	Day 14	Day 21
1.	*Penicillium*spp	1	1	2	3
2.	*Mucor*spp	3	3	4	6
3.	*Aspergillus*spp	10	9	12	17
4.	Others	7	6	5	5
	Total	21	19	23	31
	Mean	5.25	4.75	5.75	7.75

Table 5 shows the number of fungi isolated from soil samples treated with chlorpyrifos. The colony counted on Day 1 was 21×10^4cfu/g which were lower than that observed in the control sample. The colony count further reduced after Day 7 of sampling having 19×10^4cfu/g. Day 14 showed an increase in the colony count with 23×10^4cfu/g and a further increase on Day 21 with 31×10^4cfu/g which was a higher colony count than that observed on the first sampling day.

Table 6: Bacteria isolates from soil not treated with pesticide

S/N	Bacteria	Colony Count A	Colony Count B	Mean
1.	*Bacillus subtilis*	48×10^6	49×10^6	48.5×10^6
2.	*Pseudomonas aeruginosa*	7×10^6	8×10^6	7.5×10^6
3.	*Escherichia coli*	8×10^6	9×10^6	8.5×10^6
4.	*Proteus* spp	9×10^6	8×10^6	8.5×10^6
5.	Others	1.7×10^7	1.5×10^7	1.6×10^{77}
	Total	8.9×10^7	8.9×10^7	

Table 6 refers to the bacterial population in the control samples where no pesticide was applied to the soil, hence the observed bacterial density that enriches the soil through biodegradation. Total colony counted was 8.9×10^7cfu/g.

Table 7 shows the number of bacteria isolated from the soil treated with glyphosate pesticide. The colony count reduced when compared with the control sample. Although, there were slight variations in the colony counts of individual species, the total colony count remained almost constant throughout the sampling period having an average of 58.5×10^6cfu/g.

Table 7: Bacteria isolates from soil treated with glyphosate

S/N	Bacteria	Day 1	Day 7	Day 14	Day 21
1.	*Bacillus subtilis*	40×10^6	40×10^6	43×10^6	42×10^6
2.	*Pseudomonas aeruginosa*	0×10^6	0×10^6	0×10^6	4×10^6
3.	*Escherichia coli*	3×10^6	3×10^6	8×10^6	6×10^6
4.	*Proteus* spp	7×10^6	6×10^6	2×10^6	5×10^6
5.	Others	9×10^6	7×10^6	6×10^6	3×10^6
	Total	5.9×10^7	5.6×10^7	5.9×10^7	6.0×10^7
	Mean	1.1×10^7	1.1×10^7	1.2×10^7	1.2×10^7

Table 8: Bacteria isolates from soil treated with chlorpyrifos

S/N	Bacteria	Day 1	Day 7	Day 14	Day 21
1.	*Bacillus subtilis*	21 X 10^6	19 X 10^6	23 X 10^6	35 X 10^6
2.	*Pseudomonas aeruginosa*	0 X 10^6	2 X 10^6	7 X 10^6	12 X 10^6
3.	*Escherichia coli*	5 X 10^6	10 X 10^6	8 X 10^6	7 X 10^6
4.	*Proteus* spp	5 X 10^6	5 X 10^6	4 X 10^6	4 X 10^6
5.	Others	13 X 10^6	9 X 10^6	6 X 10^6	2 X 10^6
	Total	4.4 X 10^7	4.5 X 10^7	4.8 X 10^7	6.0 X 10^7
	Mean	8.8 X 10^6	9 X 10^6	9.6 X 10^6	1.2 X 10^7

Table 8 shows the number of bacteria isolated from the soil treated with chlorpyrifos pesticide.

Discussion

Tables 4 and 5 show the colony counts of fungi in glyphosate and chlorpyrifos treated soils at different sampling days. From Tables 4 and 5, the varied effects of tested pesticides were observed in the case of fungal population size. It shows that when compared with the control sample, glyphosate treated soil and chlorpyrifos treated soil experienced a decline in the colony count. It was also noted that the soil treated with chlorpyrifos experienced a greater decline in fungal colony count when compared with glyphosate treated soil. From Table 4, it was observed that the number of fungi species present in the sampled soils reduced due to injection of glyphosate. The result is consistent with the findings of [9] who observed similar decrease in colony counts of fungal species due to glyphosate application. Glyphosate application decreased viable counts of fungi to about 39%, as compared to the control soil. However, this effect was observed only after Day 1 to Day 14 after glyphosate application. On the contrary, on the fourth sampling day (Day 21), an increase in the fungal population was found in glyphosate-treated soil with the number of fungi reaching a value of 3.7×10^5cfu/g, whereas the number of fungi in control sample was 4.6×10^5cfu/g. At the end of incubation period the herbicide had no significant effect on the numbers of fungi. The probability for this happening could be that the pesticide has little or no cidal effects on fungi. It could also be possible that the pesticide was being utilized by the fungi in the soil for growth. The pesticide may have acted as nutrient to the fungi providing it with the needed carbon source for its growth.

The application of chlorpyrifos decreased the total numbers of fungi after the Day 1 and Day 7 of insecticide treatment. In contrast, 14 days after incubation, insecticide increased the fungal population but the number of this group was still significantly lower than that in the control sample. This similar trend was observed on the last sampling day which observed a further increase in the fungal population [10].On the whole, the number of fungi species was reduced from the soil due to pesticide application which may lead to poor yield on farms [11].

Tables 7 and 8 shows the colony counts of soils treated with glyphosate and chlorpyrifos. The tables show that when compared with the control sample on table 6, glyphosate treated soil and chlorpyrifos treated soil experienced a decline in the colony count. The tables also show that the soil treated with chlorpyrifos experienced a greater decline in bacterial colony count when compared with glyphosate treated soil. From Table 7, after the application of glyphosate, the bacteria population showed a decrease indicating the effect of the pesticide on bacteria in the soil sample i.e. 5.9×10^7cfu/g compared to the control sample with 8.9×10^7cfu/g.

Although individual colony counts showed increasing tendencies after some days, the total bacterial population in the glyphosate treated soil is a magnitude less than the control sample. The result is consistent with the findings of [9] who observed similar decrease in colony counts of bacterial species due to glyphosate application. *Bacillus subtilis* showed an initial decrease population after the first day of herbicide treatment and the number remained almost constant even till the last sampling day. This may be due to spore formation found among *Bacillus subtilis* which help them survive harsh conditions. This is also in line with the findings of [12] that*Pseudomonas aeruginosa* was totally extinct after the application of glyphosate to the soil and it remained that way till the last sampling day when 4×10^6cfu/g was observed.

From Table 8, due to the application of chlorpyrifos, the bacteria population showed a decrease indicating the effect of the pesticide on bacterial population in the soil sample i.e. 4.4×10^7cfu/g observed in Day 1 compared to the control sample with 8.9×10^7cfu/g. *Pseudomonas aeruginosa* went totally extinct after the application of chlorpyrifos to the soil but the number started increasing after 7 days of pesticide application with further increase experienced after subsequent weeks [13].

Based on the hypothesis that there is no significant difference between pesticide treated soil and non-treated soil, it is then observed, with a paired t-Test, the calculated values in which t=14.9 and 24.1 for glyphosate and chlorpyrifos treated soil is greater than the table value at 0.05 level of significance. Since t-calculated exceeds t-tabulated, it is concluded that there is a significant difference between the soil treated with glyphosate and chlorpyrifos and the soil without pesticide treatment [14]. This signifies that pesticide application on soil has led to the reduction of microbial population which affects the proper functioning in soil ecosystems. That indiscriminate use of pesticide might work for few years, after a while; there will be no beneficial soil microorganism to hold on to nutrient.

Conclusion

It is concluded that in spite the importance of pesticides used in agriculture, indiscriminate use of pesticide can lead to soil degradation. Although in tropical soil, persistence of pesticides in soil and their effect on density of non-target soil organisms is minimal at a normal agricultural dose; their effect is obvious at population metabolism, with change in physiological and biochemical responses. This implies that at doses, higher than prescribed dose, would alter the microbial balance of the soil sub-system. Under this, mineralization and humification processes will be very low leading to low nutritional values, hence poor crop yield that may lead to hunger and starvation.

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