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Ecotoxicological Bioassay of Agricultural Pesticides on *Nitrobacter* species

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ABSTRACT

Effect of agricultural pesticides (Attacke, DD Force, Chloview) on *Nitrobacter* spp were analyzed in soil using standard toxicological bioassay. Mortality of the test organisms expressed as median lathal concentration (LC_{50}) was used as indices to monitor toxicity. The median lethal concentration (LC_{50}) was employed to compare the toxicities of the different toxicants on the test species. The median lethal concentration (LC_{50}) of the pesticides used decreased in the following order: (Noting that the lower the LC_{50} the more toxic the toxicant): DD Force (50.64%) followed by Attacke (54.68%) and the lowest toxicity in Chloview (57.04%). Toxicant concentration of 0%, 3.125%, 6.25%, 12.5%, 25%, 50% and 75% at the exposure time of days 1, 7, 14, 21 and 28 were employed. Increase in the percentage log mortality of *Nitrobacter* spp in soil amended with three (3) pesticide during the 28 days exposure time to the pesticide concentrations was observed.. The lethal effect (death) of the test organisms could be attributed to the type, concentration, and time of exposure to the toxicant. Pesticides are toxic, disrupt enzymatic activities, affect survival, reproduction, increase individual mortality, decrease fecundity and growth, changes individual behavior such as feeding rate and decrease the overall community biomass, density and generally leads to loss of biodiversity.It is recommended that the use of chloview pesticide should be encouraged on Nigeria agricultural farm due to its low toxic effect on *Nitrobacter* spp as compared to others

Keywords: Ecotoxicological Bioassay, *Nitrobacter* spp, median lethal concentration, toxicity.

Introduction

Pesticides application contributes greatly to the pollution of the environment. These chemicals are intentionally introduced into the soil environment to control pests, in the area of agriculture. Large scale application of these pesticides produce land pollution, pesticides leaching into ground water, surface run-off to nearest water bodies, it can also be carried by wind and soil erosion, which to a great extent contributes to the dispersal of these chemicals in the environment far from the source of application. This results in the death of wildlife while some suffer damage to vital functions such as reproductive failure (Johnson *et al.*, 2001). Nitrifying bacteria are gram-negative chemoautotrophic, aerobic bacteria that oxidize ammonia to nitrate in the process known as nitrification (Willey *et al.*, 2021) In agricultural fields and farms considerable amounts of pesticides are used to increase crop production. Pesticides are borne by soil water to reservoirs and can disturb the natural ecological balance by producing toxic effects in recipient environments.

Microbial processes are important for mineral and organic matter cycling in ecological systems, with the nitrogen cycle being crucial for all organisms (Hansson *et al*., 2001; Tu, 2006). The nitrogen cycle in soil and sediment includes several microbial processes, of which biological nitrogen fixation, denitrification, and nitrification are the most studied processes (Wonk, 2001). Nitrification is a chemoautotrophic process carried out by two bacterial groups that oxidize ammonium to nitrite and nitrite to nitrate (Fenchel *et al*., 2008). This process is important to the nitrate content in soil, which is the major source of nitrogen assimilated by higher plants, thus, of considerable ecological and agricultural importance (Hansson *et al*., 2001; Tu, 2006). Nitrification has been shown to be very sensitive to chemicals disturbances, such as those produced by pesticides (Pell *et al*., 2008). Pesticide effects on nitrification can directly influence some processes such as denitrification, which is critical because it lowers eutrophication of freshwater lakes as it removes inorganic nitrogen.

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As denitrification depends upon nitrate being formed by nitrifiers, nitrification inhibition has a negative impact on the denitrification process and, consequently, on ecosystem equilibrium (Fenchel *et al*., 2008).

Some pesticides have been found to reduce the nitrifying bacterial population. Even if this is only a short-term effect (2 weeks), it can be assumed that some pesticides have an impact on bacterial populations and structures, thus, on soil micro-biodiversity (Tu, 2001). Studies performed on different soils show varied effects of different pesticides on nitrification. Negative effects such as nitrification reduction or inhibition (Tu, 2001; Vink & Van Straalen, 1999), or no inhibitory effects at all (Tu, 2005) have been observed. An increase in nitrification properties have also been demonstrated (Rangaswamy & Venkateswarlu, 2003; Das, 2007).

Materials and Methods

Study Area and Samples Collection

The experiment was carried out at the Rivers State University, Microbiology Laboratory, Port Harcourt, Nigeria Soil samples for the experiment were collected randomly with a hand-held soil auger at the surface soil (loamy clay) between the depths of 0 to 15 cm from an agricultural garden in Rivers State University, Port Harcourt. The piece of land is situated at Longitude 4^{o} 48'18.50''N and Latitude 6^{o} 58'39.12''E. Soil samples were transported in a sterile polythene bags to the microbiology laboratory for analysis.Three different brands of Agricultural pesticides (Attacke, Chloview and DD Force) were purchased from Mile 3 Market.

Microbiological Analysis

Isolation of *Nitrobacter* species

Winogradsky Agar medium composition as modified by Williams and Ogolo, (2018) was used: Agar-Agar 15.0g, $NaNO_2$ 0.05g, Na_2CO_3 1g, NaCl 0.3g, K_2HPO_4 0.5g, $MgSO_4.7H_2O$ 0.02g, $ZnCl_2$ 0.03g and $FeSO_4.6H_2O$ 0.02g were dissolved in 200ml of distilled water and autoclaved at 121^{0}C for 15 minutes at 15psi after which it was allowed to cool to about 40^{0}C and the medium was poured into Petri-dishes.

One gram (1g) of soil was mixed into 9ml of sterile distilled water and 10 fold serial dilution was done up to 10^{-2}. Aliquot (0.1ml) from appropriate dilution were inoculated onto the surface of Winogradsky agar and incubated aerobically for 2-3 days at room temperature (30 ± 2^{0}C), greyish, mucoid, flat colonies revealed pear shaped, and Gram negative, suspected *Nitrobacter*.Williams and Ogolo, (2018).

Confirmation of *Nitrobacter* species

Suspected *Nitrobacte*r species were sub-cultured onto a fresh Winogradsky agar medium and transferred into a broth containing nitrite carbonate medium and incubated at about (30 ± 2^{0}C) for 2-3 days. Five drops of Griess illosvay's reagent was added to the medium after 2days of incubation. Absence of purplish colour indicated a positive result for Nitrobacter species, further confirmation was done by addition of diphenylamine. Cherry red colour indicated presence of *Nitrobacter*.Williams and Ogolo, (2018).

Preparation Stock Solution of Pesticide

The stock solution of pesticides was prepared based on manufacturer's instruction (800ml of pesticides into 100 liters of water). The pesticide was prepared, using a volume of 8ml of pesticides transferred into 1 litre of distilled water from which the concentrations were obtained.

Toxicity Test Procedures

The pesticides were prepared aseptically by varying the concentrations as follows: 0%, 3.125%, 6.25%, 12.5%, 25% 50% and 75% of the pesticides. Using a measuring cylinder, each was aseptically transferred, that is; 3.125ml, 6.25ml, 12.5ml, 25ml, 50ml and 75ml of the different pesticides stock solution, into 96.875ml, 93.75ml, 87.5ml, 75ml, 50ml and 25ml, of sterile distilled water, respectively. The toxicity test procedures was done by using 42 sterile bowels containing 1.5kg of oven sterilized soil, 10ml of bacteria (*Nitrobacter* spp) was added separately and each pesticides concentration was added separately into the different properly labeled bowels and a control experiment was done without inoculation of pesticides and mixed using a sterile spoon. One gram of soil sample from all concentrations was serially diluted and 0.1ml aliquot from 10^{-2} to 10^{-3} dilutions was used for inoculation using spread plate techniques on Winogradsky media.

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The toxicity monitoring was done on weekly interval of: 1, 7, 14, 21 and 28, respectively, and plates were incubated for 3 to 4days at room temperature (30 ± 2^0C). Tolerance level was measured using the total viable counts (TVC) as an index, (Williams and Dilosi, 2018).

Percentage (%) Log Survival of the Bacteria

The percentage log survival of the test organisms exposed to toxicant was calculated according to the formula used by Nrior and Obire (2015).

$$Percentage\ (\%)\log survival = \frac{\text{Log c } x100}{\text{Log c}}$$

where: log c = logarithm count in each toxicant concentration,
log c = logarithm count in the control (zero toxicant concentration).

Percentage (%) Log Mortality of The Test Organisms

The percentage (%) log mortality of the test organisms was obtained using the formula adopted by Nrior and Qbire (2015) by subtracting one hundred from the value of the percentage (%) log survival. percentage (%) log mortality = 100 - % log survival.

Determination of The Median Lethal Concentration (Lc_{50})

The median lethal concentration of the toxicant was determined by subtracting the value of the highest concentration value used from the sum of concentration difference multiply by mean percentage mortality then divide by the control.

That is,

$LC_{50} = LC_{100} - (\sum$ conc. diff. × mean % mortality) / % control.

Statistical Analysis and Median Lethal Concentration (LC_{50})

Data representing % mortality and concentration from semi-static bioassay were analysed using the probit analysis software to determine the LC_{50} values in the soil.

The results obtained from toxicity screening were subjected to statistical analysis using Analysis of variance (ANOVA) and student t-test at 0.05 confidence limit (Finney,2008; Parker, 2009; Reish and Oshida, 2007) to determine the significant difference between the susceptibility of the *Nitrobacter* sp. And Nitrosomonas sp (test organism) in the different concentrations of the test toxicant. (Attacke, Chloiew and DD Force). The median lethal concentration was calculated using regression analysis (Finney, 2008).

Results

The result of the **l**ethal toxicity of Agricultural pesticides (Attacke, Chloview and DD Force) on *Nitrobacter* spp using pesticides concentration at 0%, 3.125%, 6.25%, 12.5%, 25%, 50% and 75% and monitored at an interval of 1, 7, 14, 21, and 28 days. was within the range of 41.00% - 97.90% as shown in Table 1.

The percentage (%) log survival of *Nitrobacter* sp. to (Attacke, Chloview and DD Force) pesticides

The percentage (%) log survival of the *Nitrobacter* sp. to the toxicants were shown in Fig. 1-3.The percentage log Survival of test organism *Nitrobacter* species during the 28days exposure periods to the various concentrations of the toxicants (Attacke, Chloview and DD Force) pesticides reveals that the survival rate of Chloview pesticide is higher than that of Attacke and DD Force.

Median Lethal Concentration LC_{50} Of *Nitrobacter* Spp On (Attacke, Chloview And DD Force) Pesticides.

Results of the Median Lethal Concentration LC_{50} of *Nitrobacter* spp on Agricultural pesticides (Attacke, Chloview and DD Force) are presented in Table 2. It shows the % mortality, mean % mortality, dose difference and Σ dose difference x mean % mortality. The median lethal concentration (LC_{50}) of the pesticides used, decreased in the following order: (noting the lower the LC_{50} the more toxic the toxicant): DD Force (50.64%) followed by Attacke (54.68%) and the lowest toxicity of in Chloview (57.04%).

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Table 1: Lethal toxicity of the various pesticide (Attacke, DD Force and Chloview) on *Nitrobacter* species

Incubation (Day)	Concentration (%)	Attacke + *Nitrobacter* sp.						DD Force + *Nitrobacter* sp.						Chloview + *Nitrobacter* sp.					
		3.125 %	6.25 %	12.5 %	25 %	50 %	75 %	3.125 %	6.25 %	12.5 %	25 %	50 %	75 %	3.125 %	6.25 %	12.5 %	25 %	50 %	75 %
	Control	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Day 1	% Log Survival	88.93	86.51	84.96	84.51	81.73	81.61	96.21	92.02	88.02	81.94	67.10	58.40	93.31	91.38	85.40	81.76	77.95	71.42
	% Log Mortality	11.07	13.49	15.04	15.49	18.27	18.39	6.69	8.62	11.98	18.06	32.9	41.6	6.69	8.62	14.6	18.24	22.05	28.58
Day 7	% Log Survival	90.39	87.49	85.57	82.15	76.22	73.59	94.80	92.16	87.51	79.71	65.25	56.09	91.51	89.39	84.84	83.54	75.36	69.38
	% Log Mortality	9.61	12.51	16.43	17.85	23.78	26.41	5.2	7.84	12.49	20.29	34.75	43.91	8.49	10.61	15.16	16.46	24.64	30.62
Day 14	% log Survival	92.55	88.19	76.28	74.86	71.67	65.94	94.02	91.87	87.80	83.20	56.25	46.74	92.39	88.03	80.56	75.21	70.66	67.93
	% log Mortality	7.45	11.81	23.72	25.14	28.33	36.06	5.98	8.13	12.2	16.8	43.75	53.26	7.61	11.97	19.44	24.79	29.34	32.07
Day 21	% log Survival	87.14	77.86	74.54	69.69	66.16	56.88	92.73	86.90	84.60	76.31	70.62	60.18	97.28	93.43	86.34	82.40	72.52	68.37
	% log Mortality	12.86	22.14	25.46	30.31	33.84	43.12	7.27	13.1	15.4	23.69	29.36	39.82	2.72	6.57	13.66	17.6	27.48	31.63
Day 28	% log Survival	88.63	85.22	77.15	72.44	65.55	56.18	93.70	86.98	83.27	76.62	69.26	41.11	99.00	94.35	92.34	79.03	75.27	67.49
	% log Mortality	11.37	14.78	22.85	27.56	34.45	43.82	6.3	13.02	16.73	23.38	30.74	58.89	1.00	5.65	7.66	20.97	24.73	32.51

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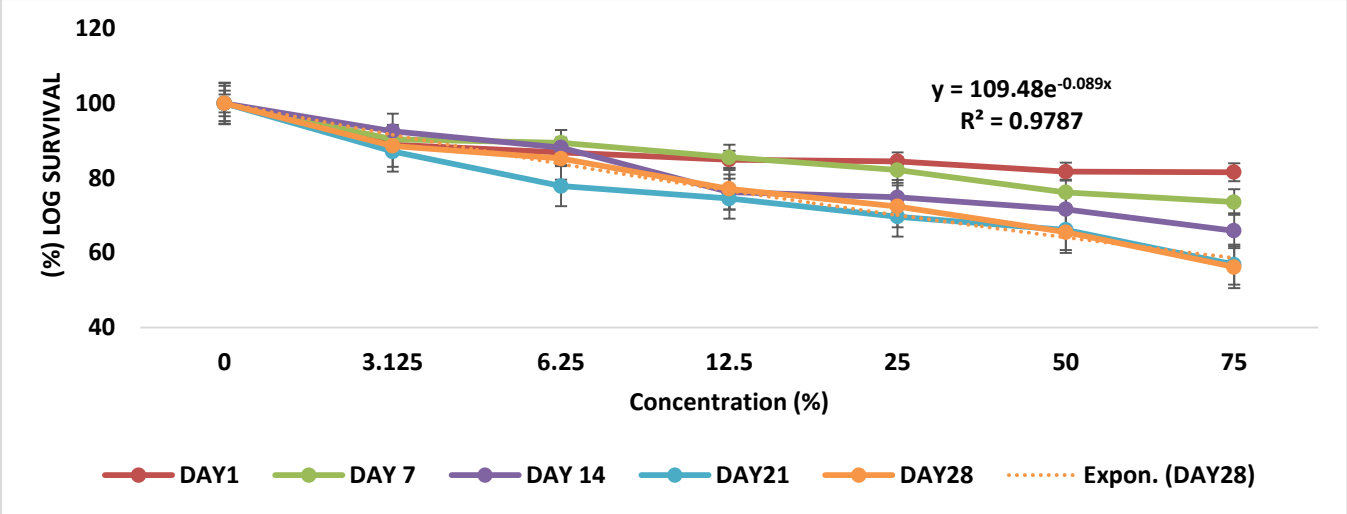

Fig 1: Percentage (%) Log Survival of Attacke pesticide on *Nitrobacter* in soil

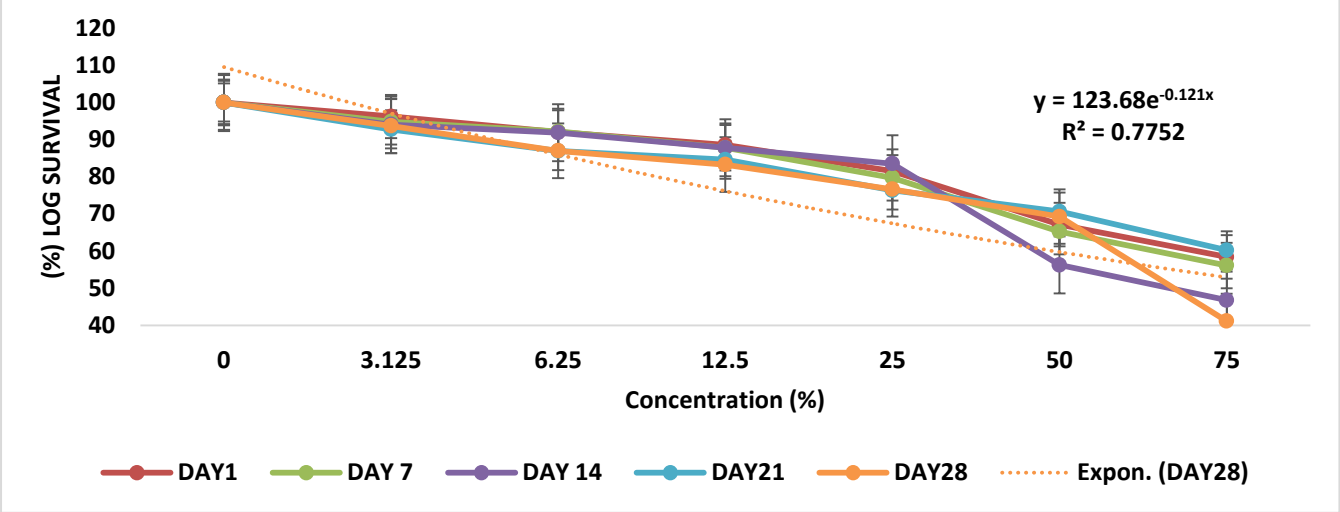

Fig. 2: Percentage (%) Log Survival of DD Force pesticide on *Nitrobacter* in soil

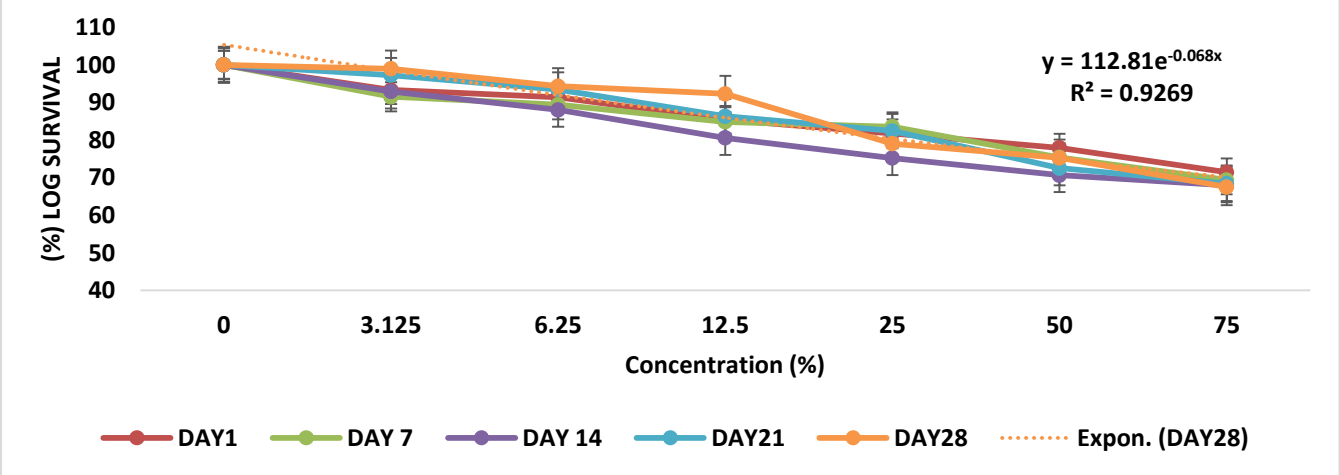

Fig. 3: Percentage (%) Log Survival of Chloview pesticide on *Nitrobacter* in soil

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Table 2: Median Lethal Concentration (LC_{50}) of the various pesticides on *Nitrobacter* species in soil

Dose		**Attacke + *Nitrobacter* sp.**			**DD Force + *Nitrobacter* sp.**				**Chloview + *Nitrobacter* sp.**			
	% mortality	**Mean % mortality**	**Dose difference (DD)**	**Σ DD × mean % mortality**	**% mortality**	**Mean % mortality**	**Dose difference (DD)**	**Σ DD × mean % mortality**	**% mortality**	**Mean % mortality**	**Dose difference (DD)**	**Σ DD × mean % mortality**
0%	-	-	-	-	-	-	-	-	-	-	-	-
3.125%	52.36	10.47	3.125	32.72	25.54	5.71	3.125	17.84	26.51	5.30	3.125	16.56
6.25%	74.73	14.95	3.125	46.72	50.07	10.01	3.125	31.28	43.42	8.68	3.125	27.13
12.5%	103.5	20.7	6.25	129.38	68.8	13.76	6.25	86	70.52	14.10	6.25	88.13
25%	116.35	23.27	12.5	290.88	102.22	20.44	12.5	255.5	98.1	19.62	12.5	245.63
50%	138.65	27.73	25	693.25	171.52	34.30	25	857.5	128.24	25.65	25	641.25
75%	167.8	33.56	25	839	237.48	47.50	25	1187.5	155.41	31.08	25	777
				Σ 2031.95				Σ 2435.62				Σ 1795.7
LC_{50}				**54.68%**				**50.64%**				**57.04%**

$$LC50 = LC100 - \frac{\Sigma \text{ Dose difference} \times \text{ mean \% mortality}}{100}$$

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Summary of 28 Days LC_{50} Pesticide (Attacke, DD Force, And Chloview) In Soil *Nitrobacte*r species in Figure 4. showed DD Force (50.64%) has the highest toxicity, followed by Attacke (54.68%) and the lowest toxicity of in Chloview (57.04%).

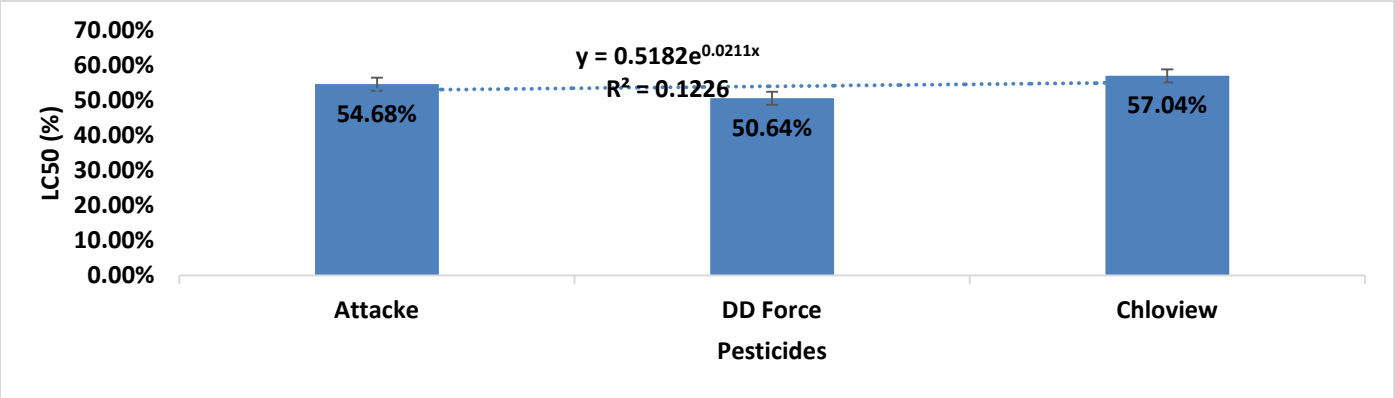

Figure 3: Summary of 28 Days (LC_{50}) of Pesticide (Attacke, DD Force, and Chloview) in soil *Nitrobacte*r species

Discussion

Toxicity test was carried out on *Nitrobacter* specie using three different brands of Agricultural pesticides (Attacke, Chloview and DD Force) also the median lethal concentration (LC_{50}) were also calculated. The lethal toxicity result of Agricultural pesticides (Attacke, Chloview and DD Force) was done by calculating the log survival of the count in each toxicant concentration divided by the log of the count in each toxicant concentration and multiply by 100. The percentage (%) log survival of the test organism *Nitrobacter* sp. to the toxicants Attacke, Chloview and DD Force pesticides were shown in Fig. 1-3. The results obtained during this research revealed that certain substances in pesticides are relatively toxic in certain concentrations to *Nitrobacter* species of bacteria. Similar observations have been reported (Wang, 2004). A good increase in the loss of *Nitrobacter* with increasing exposure time was observed in the media as the concentrations of the pesticides are increase.

During this research DD Force pesticide prove to be more lethal to *Nitrobacter* than Attacke and Chloview pesticide and the longer these organisms are being exposed to these toxicants the more lethal it becomes to them as shown in the result obtained. The percentage log mortality of *Nitrobacter* species during 28 days exposure period to soil amended with Attacke, DD Force, and Chloview pesticides (Tables 1), shows that DD Force pesticide exhibited slight effect on the test organisms than Attacke and Chloview pesticide. This may be due to the chemical composition and molecular structure of the pesticide. (Obire and Owaji-Eli, 2014,) stated that the effect of different pesticides on soil microorganisms depends on the composition of the pesticide which affects their diversity, due to their xenobiotic nature.

The percentage log Survival of *Nitrobacter* species during the 28days exposure periods to the various concentrations of the toxicants reveals that the survival rate of Chloview pesticide is higher than that of Attacke and DD Force. Hence, the results of this study reveal that DD Force pesticide caused inhibitory effect on the organisms which resulted in reduction of viable cell counts. While Attacke and Chloview pesticide was stimulatory, which means it served as carbon source for these organisms and since they were able to utilize them and proliferate which lead to increase in viable cell counts. The reduction of viable cell counts of DD Force pesticide may lead to inhibition of nitrification process (Obire and Owaji-Eli, 2014). Similar observation was done by (Das and Mukherjee, 2000) while Attacke and Chloview pesticide lead to the increase in growth of the organisms, which in turn will increase nitrification process because the organisms were able to utilize it as their sole carbon source, similar observation was done by (Williams and Dilosi, 2018).

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Nitrobacter sp. mortality expressed as median lethal concentration (LC50) was used as indices to monitor toxicity (Kpormon and Douglas, 2018). The median lethal concentration (LC_{50}) of the pesticides used, decreased in the following order: (noting the lower the LC_{50} the more toxic the toxicant): DD Force (50.64%) followed by Attacke (54.68%) and the lowest toxicity of in Chloview (57.04%).

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