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Effects of Pesticide Application on the Growth of Soil Nitrifying Bacteria

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ABSTRACT: This research studied the effects of pesticide application on the growth of soil nitrifying bacteria. *Nitrosomonas* sp. was isolated from the Soil samples gotten from five different locations within the University of Benin, Benin City and cultured using Winogradsky medium. The bacterial isolate was characterized and identified based on its cultural, morphological and biochemical characteristics. After 4 to 5 days of growth, Nitrosomonas Counts obtained from Winogradsky medium ranged from 4.8×10^3 to 9.2×10^3cfu/g. There was an increase in bacteria growth at lower pesticide concentration which began to decrease as the pesticide concentration increased with time. The percentage nitrite accumulation gradually decreased with time, the LC_{50} values were higher than EC_{50} values which shows that the bacteria could survive and grow at lower pesticide concentrations but were completely inhibited at higher pesticide concentrations. It is therefore very important to examine pesticides so as to determine its toxicity to soil bacteria before application.

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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*., 1991; Tu, 1996). 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, 1991). Nitrification is a chemoautotrophic process carried out by two bacterial groups that oxidize ammonium to nitrite and nitrite to nitrate (Fenchel *et al*., 1998). This process is important to the nitrate content in soil, which is the major source of nitrogen assimilated by higher plants and, thus, of considerable ecological and agricultural importance (Hansson *et al*., 1991; Tu, 1996). Nitrification in particular has been shown to be very sensitive to chemicals, and nitrifier activity is, therefore, often used as an indicator of chemical disturbances, such as those produced by pesticides (Pell *et al*., 1998).

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. 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*., 1998). 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 and, thus, on soil micro-biodiversity (Tu, 1991). Studies performed on different soils show varied effects of different pesticides on nitrification. Negative effects such as nitrification reduction or inhibition (Tu, 1991; 1994; Vink & Van Straalen, 1999), or no inhibitory effects at all (Tu, 1995) have been observed. An increase in nitrification properties have also been demonstrated (Rangaswamy & Venkateswarlu, 1993; Das, 1997). Furthermore, both positive and negative effects on overall metabolic activity have been found for different concentrations of a single pesticide (Laursen & Carton, 1999). The aim of this study was to evaluate the effect of pesticide application on the growth of soil nitrifying bacteria.

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MATERIALS AND METHODS

Isolation and characterization of nitrifying bacteria: Soil samples gotten from five different locations within the University of Benin, Benin City. *Nitrosomons* was isolated from the soil using the method employed by Okpokwasili and Odokuma (1996) and Colwell and Zambruski (1972), respectively. *Nitrosomonas* was isolated using Winigradsky medium for nitrification phase 1 $(NH_4)_2SO_4$, 2.0g; K_2HPO_4, 1.0g; $MgSO_4.7H_2O$, 0.5g; NaCl, 2.0g $FeSO_4.7H_2O$, 0.4g; $CaCO_3$, 0.01g, Agar 15.0g; Distilled water 1000ml. The media was sterilized by autoclaving and aseptically dispersed into sterile Petri dished after cooling to about 45^oC. The Petri dishes were then inoculated with and incubated aerobically for 4 days at room temperature (28±2^oC). Further identification and characterization of pure cultures of the organisms was undertaken. The broth media used for isolation of the test organisms also served as diluents for producing the various toxicant concentrations.

Standard inocula: Discrete colonies from each of the different plates were sub-cultured into fresh media. These were transferred into slants and stored at 4^oC. The slant culture served as stock culture. The standard inocula were prepared from the stock culture. Each of the isolate was picked from the respective stock cultures and incubated at 28^oC for 24 hours. One millimeter was transferred from the respective flasks and a ten-fold serial dilution was made up to 10^{-3}. An amount (0.1) of the 10^{-3} dilution was plated into Winigradsky agar plates. The plates were incubated at room temperature for 48 hours.

Characterization and identification of bacterial isolates: The bacterial isolate was characterized and identified based on its cultural, morphological and biochemical characteristics which includes: Gram staining, Motility Test, Catalase Test, Oxidase Test, Coagulase Test, Urease Test, Indole Test, Citrate utilization Test, Sugar fermentation Test, Nitrate Reduction (Olutiola *et al*., 1991; Holt *et al*., 1994; Cheesbrough, 2005).

Preparation of diluents for toxicity tests: Sodium nitrite (0.25mg $NaNO_2$/I Winogradsky broth) and ammonium sulphate (5.0mg $(NH_4)_2SO_4$/I Winogradsky broth) diluents for *Nitrosomonas* was employed. The diluents were sterilized at 121^oC and 15psi for 15 minutes (Atuanya *et al*., 2016)

Preparation of pesticide concentrations for Nitrosomanas toxicity test: The median effective concentration (EC_{50}) of the pesticides was determined from toxicant concentrations of 20, 40, 60, 80 and 100ppm. A control experiment consisting of the $NaNO_2$ diluent only (without toxicant) was set up (Atuanya *et al*., 2016). Insecticide concentration of 120, 140, 160, 180 and 200ppm were used for the determination of the median lethal concentration (LC_{50}). A control experiment consisting of the $(NH_4)_2SO_4$ diluent only (without toxicant) was also set up (Atuanya *et al*., 2016)

Nitrosomonas acute toxicity tests: To each of the toxicant concentrations (90ml) in 250 ml volumetric flask, 10ml of bacterial standard inoculum was aseptically inoculated. Nitrate content was determined and plates containing Winogradsky media were inoculated by spread plate technique (Okpokwasili and Odokuma, 1996). This was followed by nitrite determinations and spread plate inoculations from the various toxicant concentrations after 1, 2, 3 and 4 hours intervals (Atuanya *et al.,* 2016). Plates were incubated at room temperature (28±2^oC) for 72 hours. Percentage nitrite accumulation was plotted against toxicant concentration and the median effective concentrations (EC_{50}) were determined using the probit analysis. The percentage nitrate accumulation was plotted against time and the median lethal concentration (LC_{50}) value was calculated using the probit analysis.

RESULTS AND DISCUSSION

The results from this reseach show that the soil samples gotten from five different locations within the University of Benin all tested positive for the presence of *Nitrosomonas*. After 4-5 days of growth, the following counts were recorded on the growth medium as shown in (Table 1 and 3). Cultural, biochemical and morphological characteristics are shown in (Table 2). Table shows the percentage nitrite accumulation by nitrosomonas and (Table 5) records the LC_{50} and EC_{50} values of the test system.

Table 1: Nitrosomonas Counts in for different Soil Samples

Samples	10^{-3}	10^{-4}	10^{-5}	Mean (cfu/g)
A	76	56	21	9.2×10^3
B	168	72	26	1.2×10^4
C	37	14	09	3.6×10^3
D	119	87	33	1.4×10^4
E	63	29	11	4.8×10^3

The nitrifying bacteria *Nitrosomonas* sp. was isolated from the soil samples collected from different locations of uncontaminated agricultural soil of the University of Benin, Edo State, Nigeria. It has been observed that *Nitrosomonas* sp. have been isolated from soil samples (Ibiene and Okpokwasili, 2011). Also, isolation of *Nitrosomonas* sp. from soil

contaminated with the pesticide. The effect of different concentrations of lindane on the growth of the isolates showed that low concentrations of lindane had no inhibitory effects on the growth of the organisms within the period of the test (John and Okpokwasili, 2012), while high concentrations used in this experiment had adverse effect on the growth of the organisms as concentrations of 60ppm and above resulted in no growth witnessed.

Table 2: Cultural, Morphological and Biochemical Characteristics

Characteristics	**Description**
Shape	Round
Elevation	Raised
Margin	Entire
Wetness/dryness	Wet
Transparency	Opaque
Color	Brownish
Size	Small
Morphological	
Gram staining	-
Cell type	Rod
Cell arrangement	Single
Motility	-
Biochemical	
Catalase	+
Oxidase	-
Coagulase	-
Urease	-
Indole	-
Citrate	+
Nitrate reduction	-
Ammonium reduction	+
Sugarfermentation	
Glucose	+
Lactose	-
Possible isolates	*Nitrosomonas* sp.

Table 3: Growth of *Nitrosomonas* sp. to pesticide contaminated soil.

Conc. of Lindane (ppm)	**0 hr**	**24 hrs**	**48 hrs**	**72 hrs**
Control	9.8×10^4	1.15×10^4	1.27×10^5	1.51×10^5
20	6.4×10^4	9.2×10^4	1.07×10^4	1.29×10^4
40	1.6×10^4	3.1×10^4	5.6×10^4	4.8×10^4
60	NG	NG	NG	NG
80	NG	NG	NG	NG
100	NG	NG	NG	NG

Table 4: The percentage nitrite accumulation by *Nitrosomonas* sp.

Time (hr)	% Nitrite accumulation
1	82.16
2	56.73
3	28.91
4	11.31

Table 5: LC_{50} and EC_{50} values of the test system

Time (hr)	LC_{50}	EC_{50}
1	1973.27	80.86
2	1348.30	59.87
3	679.59	36.90
4	256.54	22.38

This shows that *Nitrosomonas* sp. can still grow at low pesticide concentrations but higher pesticide concentrations becomes toxic to the organisms. There was a decrease in nitrite accumulation at lower contaminant concentration which began to increase as the pesticide concentration increased with exposure time (Table 4). This observation is in conformity with the report of Duddleston *et al.,* (2000). The adverse effects of lindane concentrations on the nitrifying bacteria may also be attributable to the fact that the organism was subjected to a sudden shock effect of exposure to lindane concentrations as opposed to a gradual build up, since this organism was isolated from soils that have not been previously exposed to pesticide contamination. This may be part of the reason why these isolates could not grow at concentrations above 40ppm. The LC_{50} and EC_{50} values decreased with increase with exposure time for the test insecticide as observed in a study carried out by Ibiene and Okpokwasili (2011). This indicates that the toxic effect exhibited by lindane on Nitrosomonas is probably due to interference on ammonia oxidizing enzyme activity. Lang and Cai (2009) in their study observed that chlorothalonil at the field rate had a slight inhibitory effect on one soil only out of six. Conversely, chlorothalonil at higher rates inhibited nitrification significantly in all soils. Also, Lal and Saxena (1982) have reported that increasing concentrations of organochlorine insecticides reduced total viable count of marine bacteria.

*Conclusion***:** The identification of nitrifying bacterial strains with biological potentials and metabolic capacities to degrade or utilize pesticides as carbon sources is considered one of the promising approaches to enhancing soil fertility in an ecosystem contaminated with these pollutants. Thus, it is important to examine the response of these organisms to these pesticides so that less toxic and more readily biodegradable pesticides may be developed especially if the current ones in use are toxic, persistence and thus do not meet regulatory requirements in terms of their pollution effects in our environment.

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