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EVALUATION OF THE ACUTE TOXICITY OF THREE LOCALLY SYNTHESIZED DYES (3,5-DIMETHOXYPYRIMIDINE AZO-6- METHYL URAZIL, 5-ETHOXYBENZOTHIAZOLE AZOBENZOLOXYPHENOL AND 4-ETHYL 5,2,3-THIAZOLE AZO-6-METHYL URACIL) USING *NITROSOMONAS SP* AND *NITROBACTER SP*

Akponah E.[1] --- Otutu J.O.[2] --- Akpomie O.O.[3] --- Ubogu M[4]

[1,3]*Department of Microbiology, Delta State University, Abraka, Nigeria*

[2]*Department of Chemistry, Delta State University, Abraka, Nigeria*

[4]*Department of Microbiology, University of Agriculture, Makurdi, Nigeria*

ABSTRACT

The acute toxicity of three dyes (3, 5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyl of uracil were evaluated using Nitrosomonas and Nitrobacter. Standardized inoculum ofeach test organism was introduced into various logarithmic concentrations (0.01 to 100 mg/L) of the dyes for a period of 96 hours and cell mortality was used as index for assessment. Results obtained Indicated that the three dyes were inhibitory to the survival of the organisms. An increase in cell mortality (toxic response) of each organism with increase in concentration of the dyes and the duration of exposure was observed. The 96h LC50 values of 3,5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyluracil to Nitrosomonas were 2.89,2.80 and 5.30 (mg/L) respectively. Similarly, the96h LC50 values of 3,5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyl of uracil to Nitrobacter were 0.001, 0.20 and 1.11 respective;ly. Anaysis of variance at p = 0.05 showed thatthere was no significant difference in the response of Nitrosomonas to the various dyes. However, there was a significant difference in the response of Nitrobacter to the three dyes in the order 3,5-Dimethoxypyrimidine azo-6 - methyl uracil >5-Ethoxybenzothazole azobenzoloxyphenol>4-Ethyl 5,2,3-thiazole azo-6-methyl uracil. T-test also revealed that there were significant differences in the response of NitrosomonasandNitrobacterto the toxicity of each of the dye.

Keywords: Concentration, Environment, Mortality, *Nitrosomonas, Nitrobacter*, Synthesized dye**,** Toxicity.

Contribution/ Originality

This study is one of very few studies which have investigated the possible effect of locally synthesized dyes such as 3,5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyl uracil on the environment. The increased mortality of the test organisms, *Nitrosomonas* and *Nitrobacter* with increased concentrations of the dyes indicate that they are toxic to the environment.

1. INTRODUCTION

Industrial effluent contamination of natural water bodies has emerged as a major challenge in developing and densely populated countries like Nigeria [1]. Present laws and regulation on the usuage, control and discharge of chemicals in the environment requires that all commercial chemicals be assessed for their environmental behavior and potential toxicity. In Nigeria today, there is an awakening in diversification of the economy and this include the resuscitation of the textile mills in the country. This could mean an increase in the input of associated chemicals into the environment. Demands on ecotoxicological testing methodologies will therefore continue to increase as concern for environmental protection from chemical impacts increases.

Toxicity as defined by Clark [2], is a measure of how poisonous a substance is. It is the potential or capacity of a test material to cause adverse effects on living organisms. Although, the concept of toxicity appears straight forward, measuring it is subject to many complicating factors. A frequently used measure of acute toxicity is the 50% lethal dose (LD_{50}) or concentration (LC_{50}) which is the concentration of the toxicant at which 50% of organisms' population is killed.

Hitherto, the testing of the toxicity of chemicals was assessed by the use of conventional animals such as rats and mice. Other macroorganisms such as fish, snails, crabs and crayfish have also been used for toxicity bioassay [3]. However, the use of these organisms in ecotoxicological assessment was time consuming and operational cost was high. Also, there was high mortality rate due to stress of handling, feeding inadequacies and inability to adapt to experimental environment. These species therefore presented special problems that influenced their choice as test organisms. However, the preferred characteristics of an ideal bioassay testing organism were given by Williamson and Johnson [4]. These include;

i) The organism should be sensitive and convenient to use.
ii) The organism should be a representative of an ecologically important group
iii) The organisms should be widely available
iv) The organism should be genetically stable so that uniform population can be tested.
v) There should be adequate background data on the organisms including the physiology, genetics, taxonomy and role in the natural environment.
vi) The organism must also be consistent in its response to toxicants

Putting all these characteristics mentioned above into consideration, microorganisms have been recently accepted as excellent bioassay tools for ecotoxicological assessment [5]. They meet

up with the increasing demand of rapid, inexpensive and relatively simple screening test for ecotoxicological evaluation. Bacteria especially the nitrifyers are particularly important and most commonly used [6]. They play a key role in nitrogen cycle in both aquatic and terrestrial ecosystem. They have been found to be highly sensitive to a range of common environmental pollutants [5-8]. As a result this study focuses on the use of *Nitrosomonassp* and *Nitrobactersp* as the bioassay tools for the assessment of the short-term (acute) toxicity of three newly and locally synthesized dyes(3,5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyl uracil).

2. MATERIALS AND METHODS

2.1. Source of Samples (Toxicants)

The dyes used in this study were 3,5-dimethoxypyrimidine azo-6- methyl urazil, 5-ethoxybenzothiazole azobenzoloxyphenol and 4-ethyl 5,2,3-thiazole azo-6-methyl uracil. They are novel and were synthesizedlocally in the Chemistry Laboratory of Delta State University, Abraka.

2.2. Source of Test Organisms

Nitrosomonas and *Nitrobacter*used in this study were isolated from soil sample collected from a farm land in Abraka, Delta State . Surface soil samples (5-10cm depth) were collected into newly bought black polyethylene bags and transferred immediately to the laboratory for analysis.

One gram of the soil sample was measured and aseptically transferred into 9ml of sterile physiological saline contained in a test tube plugged with cotton wool. This was properly shaken and serial dilution was done up to the third dilution factor (10^3). Then 0.1ml each of these suspension were withdrawn and aseptically inoculated into Winogradsky phase I and II media using the pour plate method for the isolation of*Nitrosomonas* and *Nitrobacter*respectively. Incubation of all plates followed immediately at room temperature (27± 2°C) for 7-14 days. The colonies that developed were sub-culture and identified based on morphological characteristics and Gram reaction. Additionally, identity of *Nitrosomonas* was further confirmed by ability to accumulate nitritein broth containing NH^{4+} while that of*Nitrobacter*was confirmed by the accumulationof nitrate in broth containing NO^{2-}

2.3. Preparation of Standard Inoculum of the Isolates

Five colonies of each isolate was picked from their respective stock culture using a sterile inoculating needle and inoculated aseptically into 100ml of appropriate broth(Winogradsky phase I and II broth for *Nitrosomonas* and *Nitrobacter* respectively) contained in a 250ml conical flask. These were incubated at room temperature for 14 days afterwhich 1ml each was separately withdrawn from the respective flask and ten-fold serial dilution was done up to 10^{-6}. Further, 0.1ml of the various dilutions was inoculated into appropriate agar plates using the pour plate

technique. Incubation under appropriate conditions followed immediately (27± 2°C for 7-14 days). The dilution factor that resulted in 30-300 colonies at the end of incubation was selected as standard inoculum for the toxicity test.

2.4. Toxicity Test

Toxicity test was carried out to measure the effect of the three dyes on the cell survival of the isolates. The percent log survival test described by Williamson and Johnson, 1981 was adapted. Five logarithmic concentration of each of the test chemical which included 0.01,0.1, 1.0, 10.0 and 100.0 (mg/l) wereprepared using physiological saline. The various toxicant concentration of each dye was inoculated with 0.1ml of the standardized bacterial suspension. Incubation followed under shaken conditions using a rotary shaker at room temperature (27± 2°C) for 96 hours. Control set ups contained physiological saline and appropriate organism only. At exposure times 0h, 2h, 4h, 12h, 24h, 48h, 72h and 96h respectively, sterile pipettes were used to withdraw 0.1ml from each flask and inoculated into duplicate set of plates which contained the appropriate medium using the pour plate method. The plates were immediately incubated at room temperature (27± 2°C) for 7-14 days. At the end of incubation, counts were made and results expressed as colony forming units per milliliter ($CFUml^{-1}$). Percent log survival was calculated by dividing the log of the mean of counts in each toxicant concentration by the log of the mean of counts in the control and then multiplying by 100 Williamson and Johnson [4].

i.e. % log survival = Log C/ log c x 100

Where C is mean of counts in each toxicant concentration

cis mean of conuts in control.

The median lethal concentration (LC_{50}) which is the concentration at which 50% of the initial population of organism died at the various time of exposure was obtained from the curve of the graph of percent log survival versus toxicant concentration.

3. RESULTS

3.1. Effects of Test Dyes on Percent Log Survival of *Nitrosomonassp*

The effects of the various dyes used in the study on the percent log survival of *Nitrosomonas*spare as presented in Figs. 1 – 3. All the dyes resulted in a decrease in the log survival of the organism with increase in the exposure duration as well as with increasing toxicant concentrations.LC_{50} values obtained at various exposure hours are shown in Table 1. Also, it was noticed that theLC_{50} values decreased with increases in exposure duration. The 96h LC_{50} of 3,5-Dimethoxypyrimidine azo-6 - methyl uracil ,5-Ethoxybenzothazole azobenzoloxyphenol and 4-Ethyl 5,2,3-thiazole azo-6-methyl uracil were 2.89, 2.80 and 5.30 (mg/L) respectively.Analysis of variance at p = 0.05 showed that there was no significant difference in the response of *Nitrosomonas* to the various dyes in terms of their LC_{50} values.

3.2. Effects of Test Dyes on Percent Log Survival of *Nitrobacter*

The results of the effects of the various dyes on survival of *Nitrobacters*pare presented in Figs. 4-6. Again, the log survival values obtained reduced drastically as contact time and concentration of each dye to which the organism was exposed increased.Beyond 24h of exposure 100% mortality was observed at all exposure concentrations with exceptions of 0.01mg/l. Again, LC_{50}values as presented in Tablle 2, decreased as exposure durations increased. LC_{50}values for 3,5-Dimethoxypyrimidine azo-6 - methyl uracil ,5-Ethoxybenzothazole azobenzoloxyphenol and 4-Ethyl 5,2,3-thiazole azo-6-methyl uracil ranged from 46.40 to 0.35 mg/L, 66.59 to 0.20 (mg/L)and 5526.14 to 1.11 (mg/l) as contact time increased from2h to 96h. Anaysis of variance at p = 0.05 showed that there was a significant difference in the response of *Nitrobacter* to the three dyes in the order 3,5-Dimethoxypyrimidine azo-6 - methyl uracil >5-Ethoxybenzothazole azobenzoloxyphenol>4-Ethyl 5,2,3-thiazole azo-6-methyl uracil.T-test also revealed that there were significant differences in the response of *Nitrosomonas*and*Nitrobacter*to the toxicity of each of the dye.

Fig-1. Effect of 3,5-Dimethoxypyrimidine azo-6 - methyl uracil on the Survival of *Nitrosomonas*sp

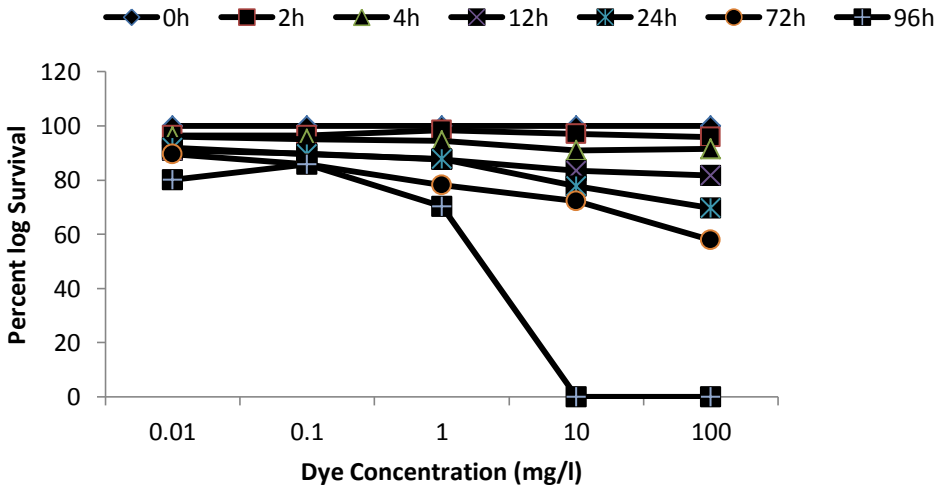

Fig-2. Effect of 5-Ethoxybenzothazole azobenzoloxyphenol on the Survival of *Nitrosomonassp*

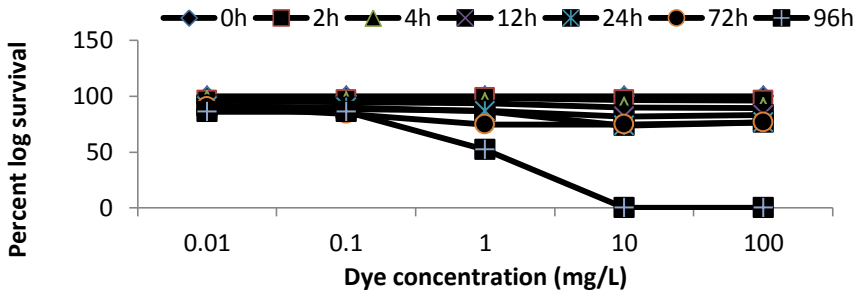

Fig-3. Effect of 4-Ethyl 5,2,3-thiazole azo-6-methyl uracil on the Survival of *Nitrosomonassp*

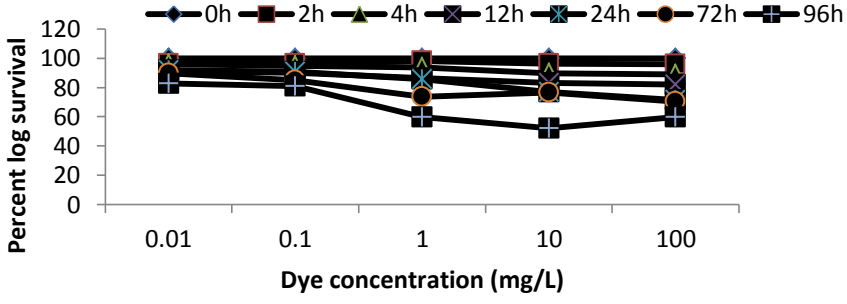

Fig-4. Effect of 3,5-Dimethoxypyrimidine azo-6 - methyl uracil on the Survival of Nitrobactersp

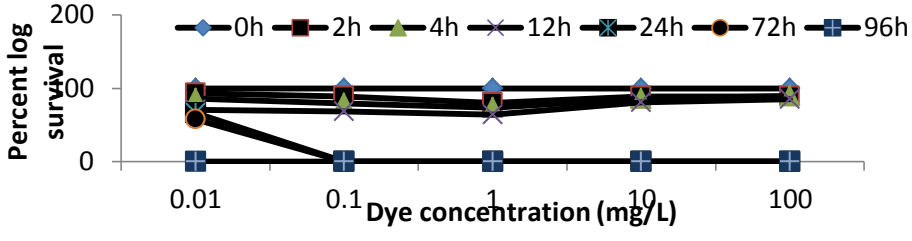

Fig-5. Effect of 5-Ethoxybenzothazole azobenzoloxyphenol on the Survival of *Nitrobactersp*

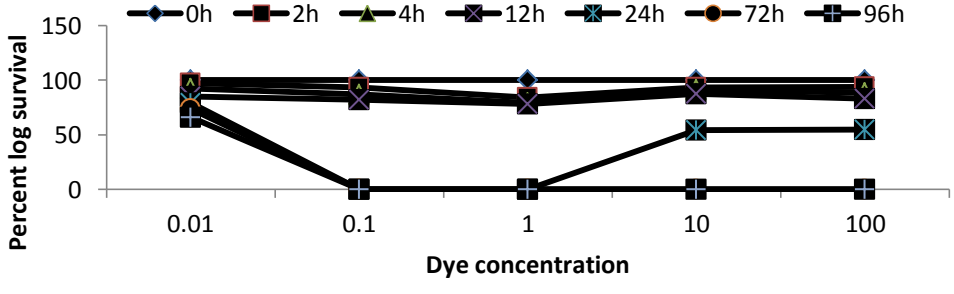

Fig-6. Effect of 4-Ethyl 5,2,3-thiazole azo-6-methyl uracil on the Survival of *Nitrosomonassp*

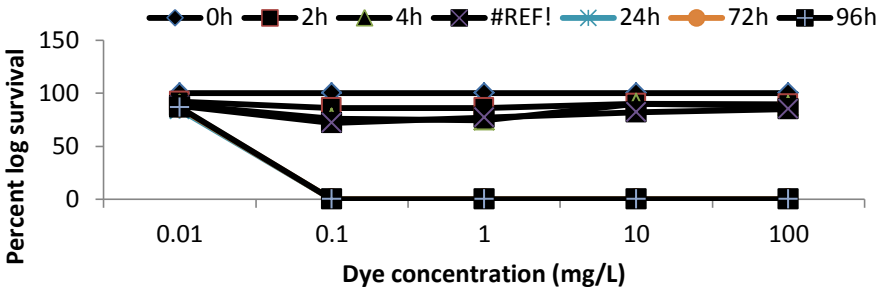

Table-1. Median lethal concentration of various dyes on *Nitrosomons*

Dye	LC50 value (mg/L)					
	2h	4h	12h	24h	72h	96h
A	104.51	36.16	17.61	8.93	6.08	2.89
B	1553.4	26.48	18.08	10.59	11.13	2.80
C	778.23	24.80	17.55	9.09	9.16	5.30

Key:A = 3,5-Dimethoxypyrimidine azo-6 - methyl uracil B = 5-Ethoxybenzothazole azobenzoloxyphenol C =4-Ethyl 5,2,3-thiazole azo-6-methyl uracil

Table-2. Median lethal concentration of various dyes on *Nitrobacter*

Dye	LC50 value(mg/L)					
	2h	**4h**	**12h**	**24h**	**72h**	**96h**
A	46.40	20.29	-2.92	0.23	0.35	0.001
B	66.59	111.22	404.68	-1.76	0.62	0.20
C	5526.14	26.84	-74.1	5.89	1.09	1.11

Key:A = 3,5-Dimethoxypyrimidine azo-6 - methyl uracil B = 5-Ethoxybenzothazole azobenzoloxyphenol C =4-Ethyl 5,2,3-thiazole azo-6-methyl uracil

4. DISCUSSION

Results obtained in this study have demonstrated the successful evaluation of the toxicity of3,5-Dimethoxypyrimidine azo-6 - methyl uracil ,5-Ethoxybenzothazole azobenzoloxyphenol and 4-Ethyl 5,2,3-thiazole azo-6-methyl uracildyes, simulating previous studies by Oranusi and Ogugbue [9] who reported the toxixity of azo dyes to *Nitrobacter*. Currently, the minimum LC_{50}value for chemicals designated as non toxic by various EPA regions is 30000ppm. In this study all LC_{50}values obtained were quite lower than the stipulated standard for non toxicity (LC_{50}> 30000 ppm) indicating the extent of toxicity of each dye. Also, the LC_{50}values of the three dyes decreased with increasing contact time. This probably arose from the fact that the dyes induced cell mortality with increases in their concentration as well as period of exposure, Similar results have been reported by Buikema, et al. [3];[10, 11]. The toxicity of the dyes tested appears to be dependent on their constituents. They all contained heavy metals such as nickel, cupper and

iron. Heavy metals have been shown tocause significant reduction in nitrification in waste water facilities [12]. Also, the aromatic ring structure of the various dyes may be contributory to their toxic actions. Thus all three dyes tested are considered relatively toxic and if disposed indiscriminately, would pose risk to the environment via short term lethal responses to organisms and hence alteration of the ecosystem especially via disruption of certain vital biogeochemical processes such as nitrification. Thus, proper measures must be taken to treat effluents containing these dyes before disposal into the environment just in case they leak into the market.

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