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INHIBITORY EFFECT OF AZO DYES ON AMMONIA-N OXIDATION BY *NITROSOMONAS*

C. J. Ogugbue and N. A. Oranusi*
DEPARTMENT OF MICROBIOLOGY,
UNIVERSITY OF PORT HARCOURT, PORT HARCOURT, NIGERIA.

ABSTRACT

The potential inhibitory effect of five azo dyes on ammonia-N oxidation was investigated. Ammonia-N oxidation was inhibited by the dyes: Mordant Black 17, Direct Red 2, Reactive Red 4, Reactive Yellow 2 and Direct Blue 14 at the concentrations ($0.01mgL^{-1}$ to $100mgL^{-1}$) tested. Inhibition of ammonia-N oxidation increased with increasing concentration of the toxicants. The EC_{50} values obtained were; 0.208 (Mordant Black 17), 1.813 (Direct Red 2), 7.926 (Reactive Yellow 2), 50.174 (Direct Blue 14) and 332.208 (Reactive Red 4). The results obtained were attributed to any/or all of the following factors: molecular size, impurities in the toxicants and metallic ions. The potential adverse ecological impact of the azo dyes on the productivity of ecosystems is discussed.

Key words: inhibitory effect, azo dyes, *Nitrosomonas*, ammonia oxidation.

INTRODUCTION

Synthetic dyes are extensively used in the textile, food, cosmetics, pharmaceutical, leather, paper printing and photographic industries (Chen *et al.* 1999; Stolz, 2001; Padmavathy *et al.*, 2003). Synthetic dyes are grouped into three major classes (Padmavathy *et al.*, 2003). Estimate of the dye lost in effluent during the dyeing process varies between 10-50% (Zollinger, 1987; Easton, 1995; Olligaard *et al.*,1999; Tan and Field, 2000) and released into the environment. The discharge of dye wastewaters into the environment is currently recognized globally as an environmental issue (Kim and Shoda, 1999; Moreira *et al.*, 2004).

Azo dyes consist of a diazotised amine coupled to aromatic amine or phenol and contain one or more azo bonds (Chen *et al.*, 1999). They constitute 60-70% of synthetic dyes with the greatest variety of colours and structures (Carliell *et al.*, 1995; Chen *et al.*,1999; Padmavathy *et al.*, 2003). This implies their widespread occurrence in wastewaters from the various industries. Some azo dyes and/or the aromatic amines which are intermediates in the manufacturing process or intermediate metabolites during microbial degradation of azo dyes are toxic, carcinogenic and/or mutagenic to various forms of life (Rafii *et al.*, 1991; Yonjie-He and Bishop, 1994; Ganesh *et al.*, 1995; FDA, 2000; Ogugbue and Oranusi 2005a, b).

Toxicity of dyes to microorganisms is an important consideration in assessing their environmental impact (Sponza, 2002). The nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) play an important role in the biogeochemical cycling of nitrogen (Atlas and Bartha, 1997). They are chemolitotrophs and have been considered ideal microbial indicators of perturbations caused by pollutants in natural environments and, hence their use in toxicity assays (Fuller and Scrow, 1997; Siciliano and Ray, 1991). *Nitrosomonas* and *Nitrobacter* have been used as target organisms for bioassays (Williamson and Johnson, 1981; Wang, 1984; Oranusi and Ogugbue, 2002; Ogugbue and Oranusi, 2005a,b).

There is steady increase in proliferation of both small scale and large scale dyestuff industries in Nigeria. These industries release their wastewaters without treatment into the environment. Most dyes pass through the most advanced technological treatment unaffected (Stolz, 2001). There is, therefore, the need to assess the potential toxicity of synthetic dyes commonly used by the various dye-stuff industries in Nigeria. This study is part of our on-going investigation on the potential toxicity of routinely used azo dyes in Nigeria on nitrifying bacteria.

The objective of this work was to determine the potential toxicity of five azo dyes on ammonia oxidation rate by *Nitrosomonas*. It is hoped that the data obtained would be of value in environmental monitoring of dye wastewaters in Nigeria.

MATERIALS AND METHODS

SOURCE OF ORGANISMS

Surface water samples (20ml) were collected in duplicate sets of 50ml sterile plastic containers

Correspondence Author: N. A. ORANUSI

from the upstream zone of Aba River located in Aba, Nigeria. The river receives textile wastewater from Aba Textile Mill, Aba, Niger Garment Factory Ltd., Aba and other related industries. Samples were transported to the laboratory in an ice chest box and cultured within 3h of collection.

MEDIUM

All chemicals used were of analytical grade. The Winogradsky medium phase II contained (gL^{-1}): $(NH_4)_2SO_4$ 2g; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.5g; NaCl 2g; $FeSO_4.7H_2O$; 0.4g; $ZnCl_2$ trace amounts and deionised water 1,000ml. The pH of medium was 7.5. Sterilization was by membrane filtration (0.2μm pore size, Acrodisc). Solid medium was prepared by adding autoclaved agar No.1 (1.5%w/v) to the broth medium.

ISOLATION OF *NITROSOMONAS*

The isolation procedure was a modification of the method adapted from Colwell and Zambruskii (1972). The target microorganisms were first enriched by inoculating 10ml of water samples into 100ml of broth contained in a triplicate set of 250ml Erlenmeyer flasks. Incubation was at 28±2°C for 4 days in the dark. The culture (10ml) was then transferred into fresh sterile 100ml broth in a triplicate set of 250ml Erlenmeyer flasks and incubated as above.

Two additional enrichments in broth as described above were carried out after which, 0.1ml of enriched culture was inoculated onto a replicate set of agar plates by spread – plate method. Plates were incubated at 28±2°C and observed for growth. Discrete mucoid colonies which developed were picked, purified by repeated streaking and Gram stained. Isolates which were Gram-negative rods were picked and presumed to be *Ntrosomonas*. Isolates were further identified by growth in nitrite-free broth. Cultures that were positive for presence of nitrite after 3 days of incubation in the dark were tentatively identified as *Nitrosomonas*. Stock cultures were prepared on Winogradsky agar slants and stored at 4°C.

INOCULUMS DEVELOPMENT AND VIABILITY TEST

Cells were transferred from stock cultures into 20ml Winogradsky broth and incubated at 28±2°C with shaking for 48h for maximum biomass yield. The cells were washed in nitrite-free physiological saline using a vortex mixer and allowed to stand for 1h. The cell sediment was resuspended in fresh sterile physiological saline and washed. The washing procedure was done repeatedly until nitrite-N was undetected thus ensuring no residual nitrite-N.

Cell viability was determined by placing 1ml inoculum into 20ml sterile ammonium sulphate solution (0.05mgL^{-1} ammonia-N) contained in duplicate set of 150ml Erlenmeyer flasks and incubated as above for 3h. Samples (1ml) of the culture were tested periodically for presence of nitrite-N. Cultures which were positive confirmed the viability of the cells and were used for bioassay.

Controls consisted of autoclaved cultures. This was to show that accumulation of nitrite-N in the experimental flasks was due to metabolic activities of the cells and not abiotic factors.

TOXICANTS

The following azo dyes – Mordant Black 17, Direct Red 2, Reactive Red 4, Reactive Yellow 2 and Direct Blue 14, were used as toxicant (Aldrich Chemical Co., U.S.A). Fig. 1 shows their chemical structures.

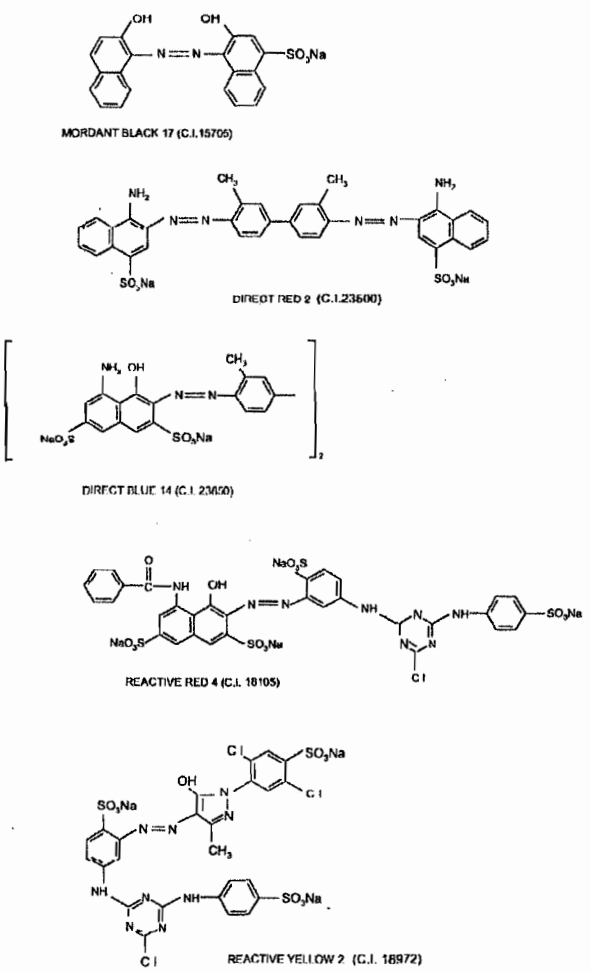

FIG. 1: STRUCTURE OF AZO DYES USED, (Adapted from Aldrich Catalogue, USA)

TEST SOLUTION

The solution was prepared by dissolving 0.32mg ammonium sulphate in 970ml deionised water and dispensed in 97ml amounts into triplicate 250ml Erlenmeyer flasks. Into each triplicate set of flasks was added the appropriate toxicant concentration (mgL^{-1}): 0.01, 0.1, 1.00, 10 and 100. Controls consisted of flasks without any toxicants added. Sterilization was by membrane filtration (0.2μm size Acrodisc).

AMMONIA OXIDATION ASSAYS

Ammonia oxidation assays were carried out on each of the triplicate set of Erlenmeyer flasks containing the toxicants. Each triplicate set of flasks and controls was inoculated with 3ml of inoculum (ca. 3.0×10^6 CFU ml^{-1}) giving a final volume of 100ml in each flask. Incubation was in the dark at 28±2°C with shaking.

Samples (1ml) were immediately withdrawn from each triplicate set of flasks for each concentration at zero time and at 2h intervals for determinations of nitrite-N accumulation and total viable count. Nitrite –N was determined by coupling diazotised sulphanilic acid with N-(1-naphthyl)-ethylenediamine (NED) dihydrochloride (Greenberg *et al.*,1985; Okpokwasili and Odokuma, 1996).

Nitrite-N accumulation was obtained from the calibration curve of absorbance against various concentration of nitrite-N. Total viable count was determined as previously described (Oranusi and Ogugbue, 2002).

Ammonia oxidation rates were calculated from the amount of accumulated nitrite–N in samples taken every 2h for 8h (Brandt *et al.*, 2001). Fifty percent effective concentration (EC_{50}) was estimated from the linear regression of the plot of ammonia oxidation rate against concentration of toxicant.

RESULTS AND DISCUSSION

The effect of the five azo dyes on ammonia oxidation by *Nitrosomonas* cells are presented in Figs. 2 - 6. The data obtained show that generally, there was decrease in ammonia oxidation rate with increasing concentration of the toxicants. For example, data obtained on ammonia oxidation rate (10^{-14} moles $cell^{-1}$ h^{-1}) at various concentrations of Mordant Black 17 were as follows: 9.20±0.54 (control); 8.03±0.54 ($0.01mgL^{-1}$); 3.22±0.97 ($0.10mgL^{-1}$); 3.04±0.21 ($1.00mgL^{-1}$); 2.27±0.42 ($10.00mgL^{-1}$) and 2.05±0.16 ($100mgL^{-1}$). Similar trend was obtained for the other toxicants at various concentrations (Figs. 2 - 6).

The inhibition of ammonia-N oxidation rate by the toxicants may be due to the inactivation of the enzymes (ammonia monooxygenase and/or hydroxylamine oxidoreductase) which mediate the oxidation of ammonia to nitrite by competitive inhibition. The decrease in ammonia oxidation rate with increasing concentration (Figs. 2 - 6) suggests competitive inhibition. Bedard and Knowles (1989) and McCarthy (1999) attributed the toxicity of various compounds on ammonia oxidizing bacteria to inhibition of ammonia monooxygenase enzyme. Similarly, inactivation of ammonia monooxygenase by acetylene was proposed to have occurred because the oxidation of the triple bond of acetylene by the enzyme would generate a reactive intermediate that would covalently bind to the active site of the enzyme (Hyman and Wood, 1985). Ogawa *et al.* (1988) attributed toxicity of azo dyes to intercalation of dye molecules between DNA strands of bacteria.

Table 1 shows the median effective concentration (EC_{50}) of the toxicants. Based on the EC_{50} values, the dyes were ranked in decreasing order of toxicity: Mordant Black 17>Direct Red 2>Reactive Yellow 2>Direct Blue 14> Reactive Red 4.

The varying degree of toxicity exerted by the toxicants may be attributed to the molecular weight and/or impurities in the dyes. The lower molecular weight dyes-Mordant Black 17 (416.39); Direct Red 2 (724.73) and Reactive Yellow 2 (872.97) exerted higher toxicity to ammonia oxidation than the higher molecular weight dyes Direct Blue 14 (995.23) and Reactive Red 4 (995.23).

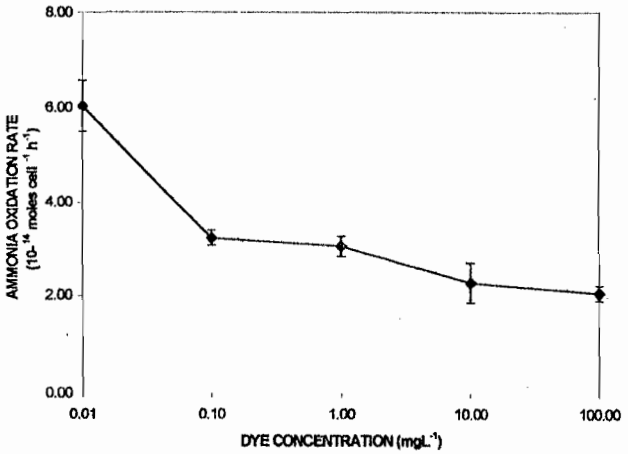

FIG. 2: EFFECT OF VARIOUS CONCENTRATIONS OF MORDANT BLACK 17 ON AMMONIA OXIDATION RATE

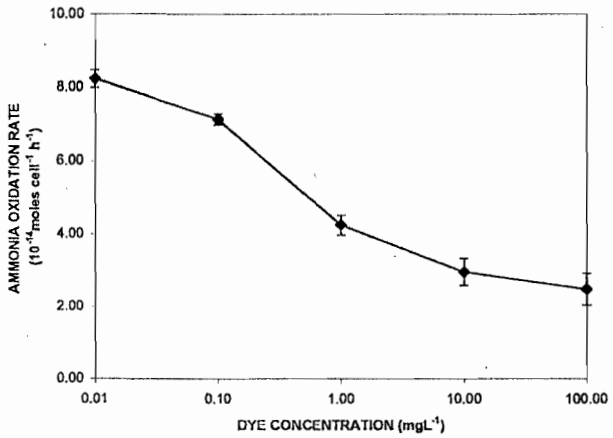

FIG. 3: EFFECT OF VARIOUS CONCENTRATIONS OF DIRECT RED 2 ON AMMONIA OXIDATION RATE OF *Nitrosomonas* SP.

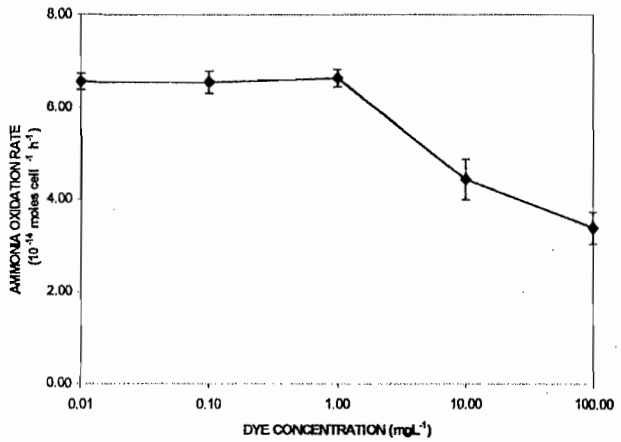

FIG. 4: EFFECT OF VARIOUS CONCENTRATIONS OPF REACTIVE RED 4 ON AMMONIA OXIDATION RATE OF *Nitrosomonas* sp.

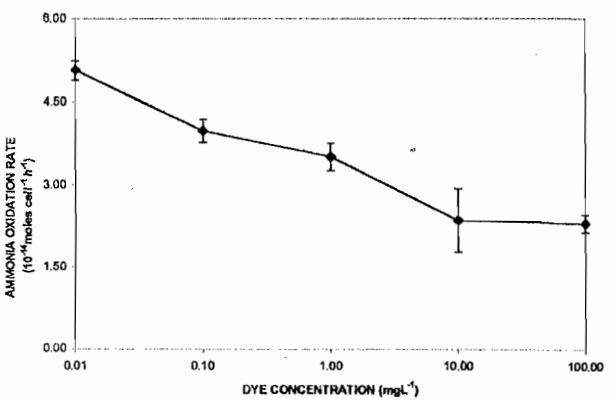

FIG. 5: EFFECT OF VARIOUS CONCENTRATIONS OF REACTIVE YELLOW 2 ON AMMONIA OXIDATION RATE OF *Nitrosomonas* sp.

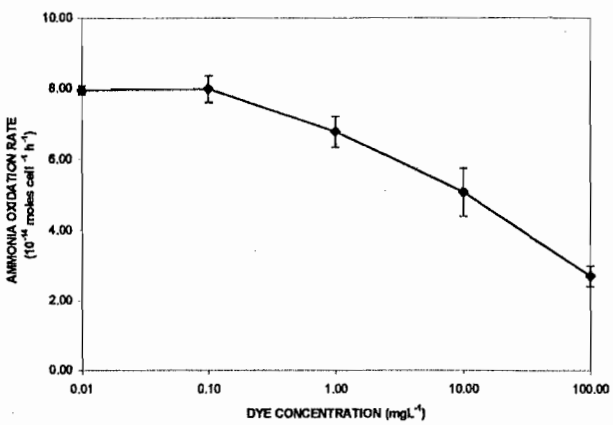

FIG. 6: EFFECT OF VARIOUS CONCENTRATIONS OF DIRECT BLUE 14 ON AMMONIA OXIDATION RATE OF *Nitrosomonas* sp.

TABLE 1 THE MEDIAN EFFECTIVE CONCENTRATION ($8HEC_{50}$)* OF FIVE AZO DYES OBTAINED WHEN *NITROSOMONAS* SP. WAS EXPOSED TO THE DYES.

Dyes Used	EC_{50} (mgL^{-1})
Mordant Black 17	0.208
Direct Red 2	1.813
Reactive Red 4	332.208
Reactive Yellow 2	7.926
Direct Blue 14	50.176

*Values were obtained from the linear regression plots of dye concentration against % inhibition.

Klassen and Eaton (1991) reported that increasing molecular weight reduces the transport of substances across cell membranes. This partially explains the differences in toxicity of the dyes based on their molecular weight.

Aromatic amines are precursor intermediates during the synthesis of azo dyes and remain as impurities in commercial azo dyes as no dye is absolutely pure (Chen *et al.*, 1999). In addition, these amines are metabolic intermediates of microbial degradation of azo dyes (Chen *et al.*, 1999; Plumb *et al.*, 2001; Yoo *et al.*, 2001). The quantity of these aromatic amines (as impurities) is propriety information and their contribution, if any, to the toxicity of the tested toxicants was not investigated. Aromatic amines have been reported to be toxic to fish (Anliker *et al.*, 1988) and to crustaceans and juvenile fish (ETAD, 1997; Oranusi *et al.*, 2002; Ogugbue and Oranusi, 2005). The aromatic amines in these toxicants may have contributed to the toxic effects of the toxicants.

Moreover, the metallic ion (Ni, Cu, Cr, Fe, Sn) content of Mordant Black 17 may have contributed to its higher toxicity compared with the other dyes.

These metallic ions are complexed in azo dyes especially the mordant dyes and act as mordant during the dyeing process. Shlegel (1992) reported heavy metal (Cu, Ag, Hg, Cr) inhibition on the activity of various enzymes even at low concentrations (oligodynamic effect). Heavy metals have also been shown to cause significant reduction in nitrification in wastewater facilities (Hu *et al.*, 2001).

The factors which contribute to the toxic effect of the toxicants (molecular weight and impurities) have been considered separately in this study, however, in reality, they may act in concert to exert the toxic effect.

This study has shown that the azo dyes tested inhibited ammonia oxidation rate of *Nitrosomonas*. These toxic effects could have serious ecological consequences on autotrophic NH_4^+ oxidation in agricultural soils and aquatic systems and on the productivity of the ecosystems. Consequent on the proliferation of textile mills in Nigeria and the large number of azo dyes currently in use, we are continuing our study on the potential toxicity of these and other azo dyes to *Nitrosomonas* and *Nitrobacter*. It is hoped that the data accumulated will assist in formulating guidelines for discharge of dye wastewater into the environment.

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REFERENCES

Anliker, R. Moser, P., and D. Poppinger. 1988. Bioaccumulation of dyestuffs and organic pigments in fish. Relationships to hydrophobicity and stearic factors. *Chemosphere.*17(8): 1631-1644.

Atlas, R.M., and R. Bartha. 1997. Biotechnological aspects of microbial ecology*In:* Microbial Ecology-Fundamental and Applications, 4th ed. AddisonWesley Longman Inc., New York. Pp. 462-590.

Brandt, K. K., M. Hesseldoe, P. Roslev, K. Henriksen, and J. Sorensen, 2001. Toxic effects of linear alkyl sulphonate on metabolic activity, growth rate, and microcolony formation of *Nitrosomonas* sp. and *Nitrospira* strains. *Appl. Environ. Microbiol.* 67(6):2498-2498.

Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C. A., Mullholand, D.A., and E. Senior. 1995. Microbial decolourisation of a reactive azo dye under anaerobic condition. *Water* SA. 21(1): 61-69.

Chen, K.C., Haug, W.T., Wu, T-Y, and Houng, J-Y. 1999. Microbial decolourisation of azo dyes by *Proteus mirabilis. J. Ind. Microbiol. Biotechnol.* 23: 686-690.

Colwell, R.R., and Zambruskii, M. S. 1972. Methods of Aquatic Microbiology. University park, Baltimore.

Easton, J. R 1995. The dye maker's view. *In:* Colour in Dyehouse Effluent, Cooper P., Society of dyers and colorists, Nottingham, Pp. 9-22.

ETAD. 1997. German ban of use of certain azo compounds in some consumer goods. *ETAD information notice* No. 6 (Revised).

FDA. 2000. Food and Drug Administration Summary of Colour Additives Listed for Use in the United States in Foods, Drugs, Cosmetics, and Medical Devices, USA.

Fuller, M. E., and K.M. Scow. 1997. Impact of trichloroethylene and toluene on nitrogen cycling in soil. *Appl. Environ. Microbiol.* 63:4015-4019.

Ganesh, R. Boardman, G.D., and Michelson, D. 1994. Fate of azo dyes in sludges. *Water Res.* 28(6): 1367-1376.

Greenberg, A.E., R.R. Trussell, and L. S. Clesceri. 1985. Standard Methods for the Examination of Water and Wastewater. Am. Public Health Assoc., Washington, DC.16th ed., Pp. 1268.

Hu, Z., Chandran, K., and B. F. Smets. 2001. Evaluation of nitrification by heavy metal nickel and zinc. WEFTEC paper, 1-15.

Hyman, M. R., and P. M. Wood. 1985. Suicidal inactivation and labeling of ammonia monooxygenase by acetylene. *Biochem. J.* 227:719-725.

Kim, S. J., and M. Shoda. 1999. Purification and characterization of a novel peroxidase from *Geotrichum candidum.* Dec. I

involved in decolourization of dyes. *Appl. Environ. Micrbiol.* 65:1029-1035.

Klassen, C. D., and D.L. Eaton. 1991. Principles of Toxicity In Cassaret and Doull's toxicity *In:* The Basic Science of Poison 4th ed. Pergamon Press, Elmsford, New York pp.12-50.

Moreira, T.A., Viacava, C. and V. Gladys. 2004. Fed-batch decolourisation of Poly R-478 by *Tametes versicolor. Braz. Arch. Biol. Technol.* 47(2): 179-183.

Ogawa, T., Shibata, M., Yatome, C. and E. Idaka, 1988. Growth inhibition of *Bacillus subtilis* by basic dyes. *Bull. Environ. Contam. Toxicol.* 40:545-452.

Ogugbue C. J., and N.A Oranusi. 2005a. Quantal response of freshwater shrimp (*Desmocaris trispinosa*) to toxicity of azo dyes. *Global J. Environ. Sci.* 4:41-45.

Ogugbue C. J., and N.A Oranusi. 2005b. Toxic effect of azo dyes on nitrite-N utilization by *Nitrobacter. Global J. Environ. Sci.* 4:47-51.

Okpokwasili, G.C., and L.O. Odokuma. 1996. Tolerance of *Nitrobacter* to toxicity of hydrocarbon fuels. *J. Petroleum Sci. Eng.* 16:89-93.

Olligaard, H., Frost, L., Galster, J., and O. C. Hansen. 1999. Survey of azo colourant in Denmark, Miljoproject. 509 Danish EPA.

Oranusi, N.A., and C.J Ogugbue. 2002. Toxic response of *Nitrobacter* to Orange II and Direct Blue 71. *J. Appl. Sci. Environ. Manage.* 6(1):87-90.

Oranusi, N. A., C.J Ogugbue, and O. Akaranta. 2002. Toxicity of Azo Dyes (Orange II and Direct Blue 71) and Sulphanilic acid to Shrimp (*Paleomonetes africanus*). *African J. Appl. Zool. Environ. Biol.* 4: 77-81.

Padmavathy, S., Sandhya, S., Swaminathan, K., Subrahmanyam, Y.V., T. Chakrabarti, T., and S.N. Kaul 2003. Aerobic Decolourisation of Reactive Azo Dyes in Presence of varius Cosubstrates. *Chem. Biochem. Eng. Q.* 17 (2):147-151.

Plumb, J.J., Bell, J. and D. C Stuckey. 2001. Microbial population associated with treatment of an industrial dye effluent in an anaerobic baffled reactor. *Appl. Environ. Microbiol.* 67 (7): 3226-3235.

Rafii, F., Franklin, W., and C. E Cerniglia. 1990. Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. *Appl. Environ. Microbiol.* 56:2146-2151.

Shlegel, H. G. 1992. Growth and inhibition of microorganisms *In:* General Microbiology. 4th ed. Cambridge University Press. Pp. 223.

Siciliano, S.D., and Ray, R. 1991. The role of soil microbial tests in ecological risk assessment: differentiating between exposure and effect. *Hum. Ecol. Risk Assess.* 5:671-682.

Sponza, D. T. 2002. Necessity of toxicity assessment in Turkish industrial discharges (examples from metal and textile industry effluents) *Environ. Monit. Assess.* 73(1):41-66.

Stolz, A. 2001. Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.* 56:69-80.

Tan, N.C.G., and J. A. Field. 2000. Biodegradation of sulfonated aromatic compounds. *In:* Environmental Technologies to Treat Sulfur Pollution: Principles and Engineering. Lens, P. and Hulshoff Pol, L. (eds.), IWA publishing, London, Pp. 373-392.

Wang, W. 1984. Time response of *Ntrobacter* to toxicity. *Environ. International.* 10:21-26.

Williamson, K.J., and D. G. Johnson.1981. A bacterial bioassay assessment of wastewater toxicity. *Water Res.* 15:383-390.

Yonjie-He, and P.L. Bishop. 1994. Effect of Acid Orange 7 on nitrification process. *J. Environ. Eng.* 120: 108-121.

Yoo, E.S., J. Libra, and L. Adrain. 2001. Mechanism of decolourisation of azo dyes in anaerobic mixed culture. *J. Environ. Eng.* 127 (9): 844-849.

Zollinger, H. 1987. Synthesis, properties and applications of organic dyes and pigments. *In:* Color Chemistry, 2nd ed., VCH Publisher, Weinheim, Pp. 92-102