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TOXIC EFFECT OF AZO DYES ON NITRITE-N UTILIZATION BY *NITROBACTER*

C. J. OGUGBUE and N. A. ORANUSI

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ABSTRACT

The toxicity of five azo dyes (toxicants) on *Nitrobacter* was investigated. The index for toxicity was inhibition of rate of nitrite-N utilization. The rate of nitrite-N utilization decreased with increase in concentration of each toxicant at specific exposure time. However, stimulation of utilization was obtained at low concentrations and short exposure time for two of the dyes. This was attributed to hyperactivity. The median effective concentration$_{50}$ (EC_{50}) values increased with increase in exposure time for each toxicant. This was attributed to acclimatization and/or detoxification. Inhibition was attributed to any/or all of the following factors: molecular size, impurities in the toxicants and dye content.

KEY WORDS: Toxicity, Azo dyes, *Nitrobacter*, hyperactivity, acclimatization

INTRODUCTION

Azo dyes are the largest class of synthetic dyes with the greatest variety of colours. At least 3,000 different varieties of azo dyes are extensively used in the textile, paper, food, cosmetics and pharmaceutical industries (Chen *et al.*, 1999). Several azo dyes are potentially toxic, mutagenic and carcinogenic which may be due to the dye itself and/or aromatic amines. These aromatic amines are derivatives generated during the reductive cleavage of the azo linkage (-N=N-) or as intermediates in the manufacturing process (Dawson, 1981; NIOSH, 1980; Young and Yu, 1997; Houk *et al.*, 1991; Brown and DeVito, 1993; Rafii and Cerniglia, 1995). Azo dyes are second only to polymers in terms of new compounds submitted for registration in the U.S.A. under the Toxic Substances Control Act (Brown and DeVito, 1993)

In textile industries, up to 50% of dyes are lost in effluents (Moreira *et al.*,2004) and pollution by dye wastewater is becoming increasingly alarming (Padmavathy *et al.*, 2003) and has become an environmental concern (Moreira *et al.*, 2004)

Toxicity of some dyes to various forms of life has been reported for Mysid shrimps (Reife, 1991); Japanese medaka (Allison and Morita, 1995); catfish (Crepsi and Cegarra, 1980) and *Paleomonetes africanus* (Oranusi *et al.*, 2002)

Aba River is located at Aba in southeastern Nigeria and is the main recipient of dye wastewater from Aba Textile Mill and other textile mills. Some evidence of pollution of the river by the dye wastewater include obnoxious odour, colouring of the river, eutrophication with attendant ecological problems (personal observation)

Nitrification is extremely sensitive to environmental stress and is one of the most sensitive microbial processes in aquatic and terrestrial environments (Stainer *et al.*, 1992). A pollutant that does not inhibit nitrification may probably not affect other processes under pollutant stress (Boyd, 1988). *Nitrosomonas* and *Nitrobacter* have been used as target organisms for bioassay (Williamson and Johnson, 1981; Wang and Reed, 1983; Wang, 1984). Bioassays using these organisms rely on quantifying the effect of the toxicants on the rate of nitrite utilisation or nitrate production for *Nitrobacter* or nitrite production or ammonium oxidation for *Nitrosomonas* (Williamson and Johnson, 1981)

Most toxicological research in Nigeria has centered on crude oil and various products of crude oil refining or chemicals used in exploration and/or exploitation of crude oil (Okpokwasili and Odokuma, 1994; Okpokwasili and Odokuma, 1996; Odokuma and Ikpe, 2003; Odokuma and Kindzeka, 2003). Our search through the literature shows that there is little or no information on the potential toxicity of routinely used dyes in Nigeria on *Nitrobacter*. This organism plays an important role on the nitrogen cycle and productivity of aquatic and terrestrial ecosystems.

The aim of this study was to assess the potential toxicity of five azo dyes commonly used by both local and large-scale textile industries on nitrite-N utilization by *Nitrobacter*. It is hoped that the results will contribute to our awareness on the ecological impact of discharge of dye wastewaters into the environment

MATERIALS AND METHOD

Source of organisms

Aba River located in Aba, Nigeria, receives textile wastewater from Aba Textile Mill, Aba, Nigeria. Surface water samples (20ml) were collected in duplicate sets of 50ml sterile plastic containers and cultured within 3h of collection.

Winogradsky medium

All chemicals were of analytical grade. The medium contained (gl^{-1}):
K_2PO_4 0.5, NaCl 0.3, $MgSO_4.7H_2O$ 0.03, $MnSO_4.H_2O$ 0.02, $FeSO_4.6H_2O$ 0.03, $NaNO_2$ 0.05, deionised water 1,000ml. The pH 7.0 .Sterilization was by membrane filteration (0.2μm pore size Acrodisc). Solid medium was prepared by adding autoclaved agar No.1 (Oxoid) at 1.5 (w/v) to the broth medium.

Isolation

The method was a modification of the method described by Colwell and Zambruskii (1972). Microorganisms were first enriched by inoculating 10ml of water sample into 100ml of broth contained in replicate set of 250ml Erlenmeyer flasks. Incubation was at 30±2°C for 4days in the dark. The culture (10ml) was then transferred in fresh sterile 100ml broth in triplicate set of 250ml Erlenmeyer flasks and incubated as above.

One milliliter of culture was inoculated onto agar plates by spread-plate method. Plates were incubated at 30±2°C and observed for growth. Greyish, mucoid and flat colonies were picked and Gram stained. Isolates which were Gram negative and pear-shaped indicative of *Nitrobacter* (Colwell and Zambruskii, 1972) were picked and purified

C. J. OGUGBUE, Department of Microbiology, University of Port Harcourt, Port Harcourt, Nigeria.
e-mail: ceejay55us@yahoo.com
✉ N. A. ORANUSI, Department of Microbiology, Faculty of Science, University of Port Harcourt, Port Harcourt, Nigeria.

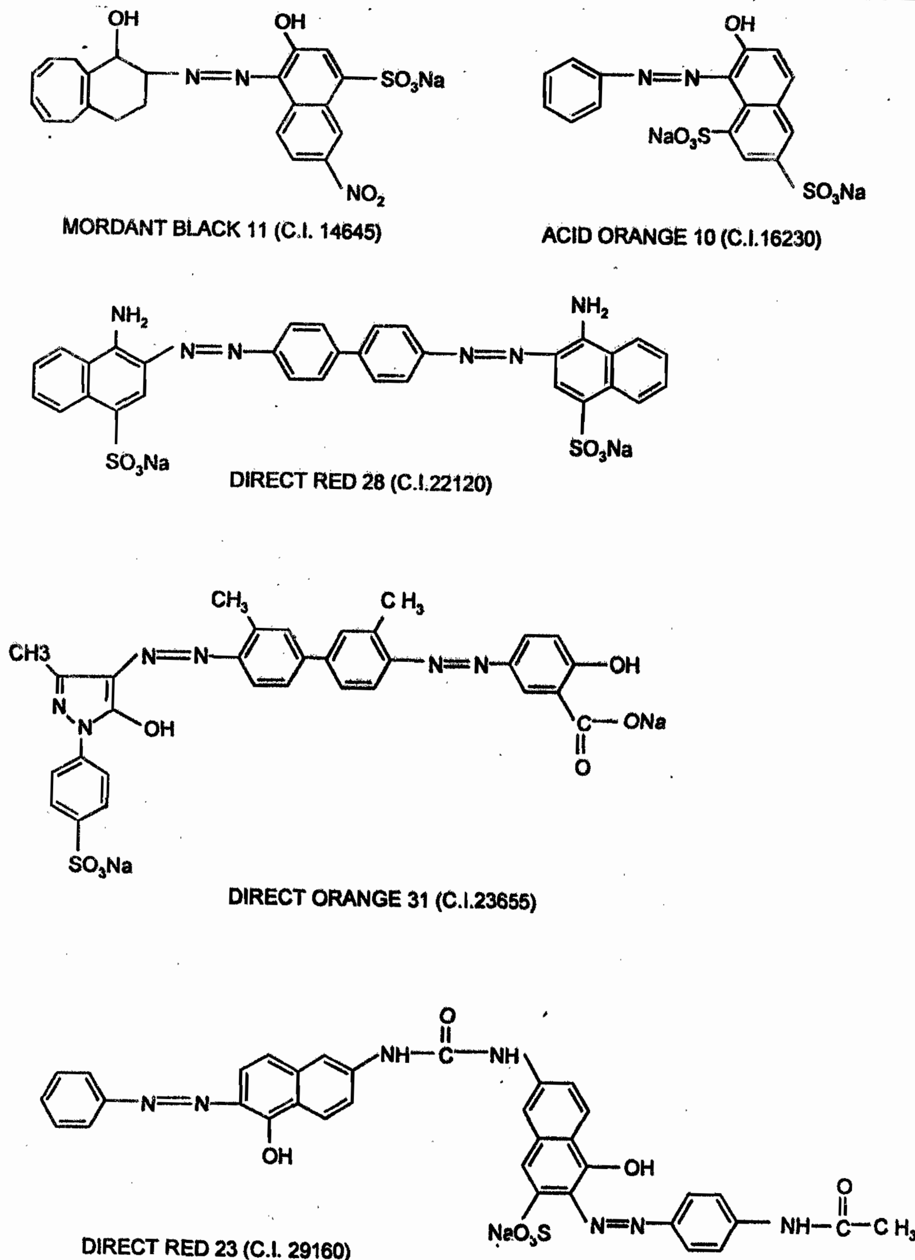

FIG. 1 STRUCTURES OF AZO DYES USED

further by repeated subculture and staining.

Stock culture in Winogradsky agar slants were preserved at 4°C in a refrigerator.

Inoculum Development and Viability Test

Colonies were transferred from stock cultures into 20ml of Winogradsky broth and incubated at 30±2°C with shaking for 36h for maximum biomass yield. Cells were suspended in sterile physiological saline, shaken in vortex mixer and allowed to stand for 1h. The cell sediment was resuspended in fresh sterile physiological saline. The procedure was repeated until nitrite-N was undetected thus ensuring no residual nitrite-N.

The viability of the culture was tested by inoculating 1ml of the inoculum into 20ml of sterile nitrite solution (0.05mgl^{-1} Nitrite-N) contained in duplicate set of 250ml Erlenmeyer flasks. Flasks were at incubated at 30±2°C for 1h with shaking. Nitrite-N was not detected after incubation in the dark. This shows that the organisms were still viable and were used for bioassay.

Controls consisted of 1ml autoclaved culture inoculated and incubated as for the sample flasks. Nitrite-N was detected after 1h. This shows that the disappearance of nitrite-N in the sample flasks (unautoclaved culture) was due to metabolic activities of the cells and not due to abiotic factors.

Toxicants

The azo dyes used as toxicants were Mordant Black 11, Acid Orange 10, Direct Red 28, Direct Orange 31 and Direct Red 23 (Aldrich Chemical catalogue, U.S.A.). Figure 1 shows the structures of the dyes.

Nitrite Solution

Nitrite solution was prepared by dissolving 0.25mg of sodium nitrite in 970ml of deionised and dispensed in 97ml amounts into 250ml Erlenmeyer flasks. Into a triplicate set of flasks was added the appropriate toxicant concentration (0.01mg, 0.10mg, 1.00mg, 10.00mg and 100mg). Controls consisted of flasks without any toxicants added. Sterilization was by membrane filteration (0.2µm pore size Acrodisc) as autoclaving resulted in precipitate formation.

Nitrite Utilization Test

Into each of the triplicate set of each toxicant concentration and controls was inoculated with 3ml of

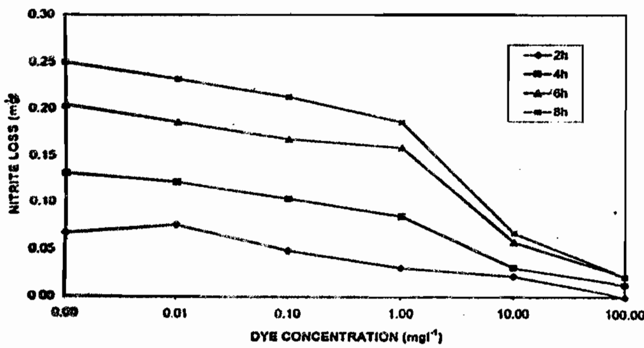

Fig. 2 Effect of various concentrations of Mordant Black 11 on nitrite utilization by *Nitrobacter* sp.

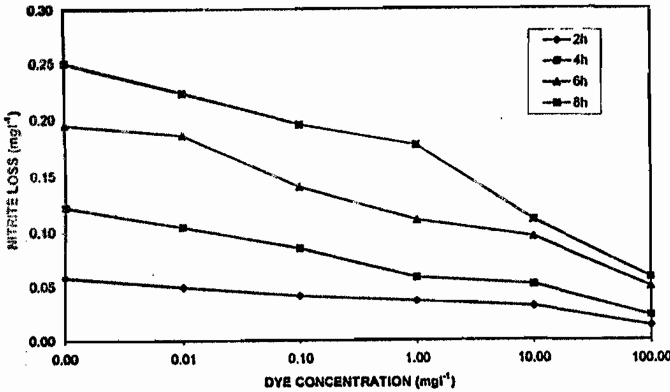

Fig. 3 Effect of various concentrations of Acid Orange 10 on nitrite utilization by *Nitrobacter* sp.

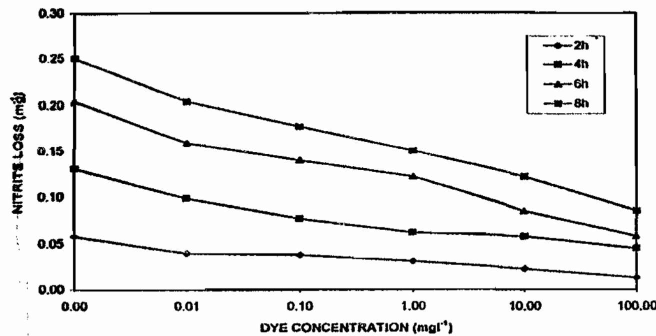

Fig. 4 Effect of various concentrations of Direct Red 28 on nitrite utilization by *Nitrobacter* sp.

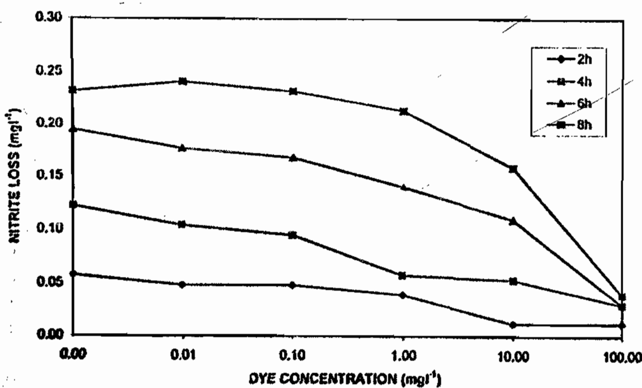

Fig. 5 Effect of various concentrations of Direct Orange 31 on nitrite utilization by *Nitrobacter* sp.

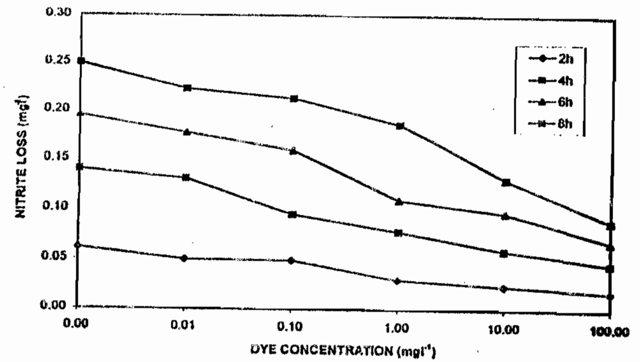

Fig. 6 Effect of various concentrations of Direct Red 23 on nitrite utilization by *Nitrobacter* sp.

inoculum (ca. 2.0 x 10^6 $CFUml^{-1}$) to give final volume of 100ml per flask. Samples (1ml) were immediately withdrawn from each flask (i.e 3ml per toxicant concentration) at zero hour and at 2h intervals for determination of nitrite-N by coupling diazotized sulphanilic acid with N-(1-naphthyl)-ethylenediamine (NED) dihydrochloride (Greenberg *et al.*, 1985 and Okpokwasili and Odokuma, 1996). Nitrite loss was determined from the calibration curve (absorbance against nitrite concentration)

RESULTS AND DISCUSSION

Figures 2 - 6 depict the results obtained on toxic effect of five azo dyes (toxicants) on nitrite-N utilization by *Nitrobacter*. Generally, nitrite-N utilization decreased with increasing toxicant concentration for same exposure time. For example, at 4h exposure time for Mordant Black 11 (Fig. 2) loss of nitrite-N (nitrite-N utilization) at various concentrations (mgl^{-1}) were as follows: 0.01mgl^{-1}(0.122); 0.10 mgl^{-1} (0.104); 1.0 mgl^{-1} (0.085); 10 mgl^{-1} (0.031) and 100 mgl^{-1} (0.013).The same trend was observed for other toxicants (Figs 3-6).

Slight stimulation of nitrite utilization was recorded at 0.01mgl^{-1} at 2h exposure time for Mordant Black 11 (Fig. 2) and Direct Red 23 (Fig. 5). This may be attributed to hyperactivity of the cells as nitrite-N is the sole source of energy (increase in metabolic activity of cells under stress to cope with toxic effect of toxicant at low toxic concentrations and short exposure time). Hyperactivity was advanced by other researchers to explain the stimulatory effect of toxicants such as cadmium and nickel toxicity on *Nitrobacter* (Wang, 1984); toxicity of toxic substances to fish (Reed *et al.*, 1980) and increased respiration of methanogenic culture on nickel toxicity (Speece *et al.*, 1983)

In the controls, nitrite-N utilization (loss of nitrite-N) increased with increase in exposure time with no residual nitrite-N at the 8h exposure time.

Time is an important factor in bioassays. There was increase in nitrite-N utilization with exposure time (Figs. 2 - 6) though at lower rate compared to the controls (no toxicants). For example, data obtained for loss of nitrite-N (mgl^{-1}) at various exposure times for Direct Red 28 at 10.00mgl^{-1} concentration (Fig.3) were as follows: 2h (0.023); 4h (0.058); 6h (0.085) and 8h (0.122). This is due to accumulation with time.

Table 1 shows the Median Effective Concentration (EC_{50}) of the toxicants. Based on EC_{50} values, the dyes were ranked in decreasing order of toxicity: Mordant Black 11> Acid Orange 10 >Direct Red 28 > Direct Orange 31 > Direct Red 23. EC_{50} values increased with increase in exposure time for each toxicant. The data may be attributed to acclimatization and/or detoxification. Wang (1980) attributed increase in EC_{50} of heavy metals on *Nitrobacter* to detoxification.

TABLE 1 The Median Effective Concentration (EC_{50})* values obtained for the five dyes

Dyes Used	EC_{50} (mgl^{-1}) 2h	4h	6h	8h
Mordant Black 11	1.31	1.55	2.39	2.70
Acid Orange 10	0.26	2.19	3.80	4.62
Direct Red 28	1.00	1.63	2.63	5.91
Direct Orange 31	2.25	2.81	7.55	12.44
Direct Red 23	2.60	3.74	7.62	15.38

*Values were obtained from the linear regression plots of dye concentration against loss of nitrite (% of control)

The varying degrees of toxicity of the toxicants may be attributed to any/or all of the following: molecular weight, impurities in the dyes and dye content. The lower molecular weight dyes: Mordant Black 11 (461.39) and Acid Orange 10 (452.38) were more toxic to nitrite-N utilization than the higher molecular weight toxicants: Direct Red 28 (696.67); Direct Orange 31 (670.62) and Direct Red 23 (813.74). Klassen and Eaton (1991) report that increasing molecular weight reduces the transport of substances through cell membranes.

Aromatic amines are intermediates in the manufacture of azo dyes (Chen *et al.*, 1999) and are impurities in commercial azo dyes. These aromatic amines have been reported to be highly toxic to fish (Anliker *et al.*, 1988) and to crustaceans and juvenile fish (ETAD, 1997). The quantity of these aromatic amines in commercial dyes is under patent protection and as such, the contribution if any, of the impurities in these dyes was not investigated. However, it is most likely that the impurities may have contributed to the toxic effects of the toxicants.

According to Aldrich Chemical Catalogue (USA), the dye content of azo dyes tested were as follows : Mordant Black 11 (80%); Direct Red 28 (85%); Acid Orange 10 (95%); Direct Orange 31 (60%) and Direct Red 23 (30%). Aromatic amines which are intermediates in the manufacturing process of azo dyes, constitute the remaining. The dyes with high dye content were more toxic than the dyes with low dye content (Figs. 2 - 6) and Table 1. However, the higher toxicity of Mordant Black11 compared with Acid Orange10 and Direct Red 28 with higher dye content may be attributed to its content of metallic ions. Mordant dyes contain metallic ions (copper, iron, chromium and aluminium) as mordants. These metallic ions have been reported (Shlegel, 1992) to inhibit the activity of various enzymes even at very low concentrations (oligodynamic effect). Zinc and copper inhibited nitrite-N consumption by *Nitrobacter* (Wang, 1984), while heavy metals inhibited nitrification in textile treatment facilities (Hu *et al.*, 2001).

The factors which contribute to the toxic effect of the toxicants (molecular weight, impurities and dye content) 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 nitrite-N utilization by *Nitrobacter*. Inhibition of nitrite-N utilization will have adverse ecological consequences on the nitrogen cycle and productivity of the ecosystem. In view of the large number of azo dyes currently in use, we are continuing our study on the potential toxicity of these and other azo dyes on *Nitrosomonas* and *Nitrobacter*.

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