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Effect of azo dyes on nitrite assimilation by *Nitrobacter*

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ABSTRACT

Five azo dyes were tested for their toxic effects on nitrite-N assimilation by *Nitrobacter* sp. Nitrite-N assimilation was inhibited by the azo dyes tested: Mordant Black 17; Direct Red 2; Direct Blue 14; Reactive Red 4 and Reactive Yellow 2 at various concentrations ($0.01 mgL^{-1}$ - $100.00 mgL^{-1}$). Statistical analysis (ANOVA) showed that there was significant difference between values obtained as nitrite-N assimilation for various concentrations of each dye at specific exposure times. However, stimulation effects on assimilation (when compared with control) were obtained at low concentrations and short exposure time with Reactive Red 4 and Reactive Yellow 2. This was attributed to hyperactivity caused by increase in metabolic activity of cells under stress to cope with toxic effects of the toxicants at sub-lethal concentrations. The ranges of EC_{50} values (mgL^{-1}) obtained at various exposure times were: Mordant Black 17 (0.375-0.965); Direct Red 2 (0.379-2.078); Direct Blue 14 (0.141-11.00); Reactive Red 4 (323.97-6455.50) and Reactive Yellow 2 (23.41-116.01). Differences in degree of toxicity exerted were attributed to any/or all of the following factors: molecular size and impurities in the toxicants. Generally, EC_{50} values increased with increase in exposure time for each toxicant and this trend was attributed to acclimatization and/or detoxification.

Keywords: toxicity, azo dyes, assimilation, *Nitrobacter*

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. Aromatic amines are generated during the reductive cleavage of the azo linkage (-N=N-) or as intermediates in the manufacturing process (NIOSH, 1980; Dawson, 1981; Houk *et al*., 1991; Brown and DeVito, 1993; Rafii and Cerniglia, 1995; Young and Yu, 1997). 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). This demonstrates their importance and application.

In textile industries, up to 50% of dyes are lost in effluents (Moreira *et al*., 2004). 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. These include: Mysid shrimps (Reife, 1991); Japanese medaka (Allison and Morita, 1995); catfish (Crepsi and Cegarra, 1980) and *Paleamonetes 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 downstream of the river. Evidence of pollution of the river by the dye wastewater includes obnoxious odour, colouring of the river and eutrophication with attendant ecological problems.

Nitrification is extremely sensitive to environmental stress and is one of the most sensitive microbial processes in aquatic and terrestrial environments (Stanier *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 assimilation or nitrate production for *Nitrobacter* or nitrite production or ammonium oxidation for *Nitrosomonas* (Williamson and Johnson, 1991).

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). There is little or no information from literature on the toxicity of routinely used dyes in Nigeria on *Nitrobacter*. Yet, this organism plays an important role in the nitrogen cycle and productivity of aquatic and terrestrial eco-

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systems.

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

MATERIALS AND METHOD

Source of organisms

Aba River located in Aba, Abia State, Nigeria, receives textile wastewater from Aba Textile Mill, Aba, Nigeria and other small textile mills downstream of the river. Surface water samples (20ml) were collected in duplicate using 50ml sterile plastic containers and cultured within 3h of collection.

Toxicants

The azo dyes used as toxicants were Mordant Black 17, Direct Red 2, Direct Blue 14, Reactive Red 4 and Reactive Yellow 2 (Aldrich Chemical Co., U.S.A.) (Fig. 1).

Winogradskii medium

All chemicals were of analytical grade. The medium contained (gL^{-1}): K_2HPO_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 was 7.0. Sterilization was by membrane filteration (0.2µm pore size Acrodisc, France). Solid medium was prepared by adding autoclaved agar No.1 (Oxoid) at 1.5 (w/v) to the broth medium.

Isolation of Nitrobacter

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 4 days in the dark in the incubator. The culture (10ml) was then transferred to fresh sterile 100ml broth into 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 colonies. Greyish, mucoid and flat colonies were picked and Gram stained. Isolates which were Gram negative and pear-shaped were indicative of *Nitrobacter* (Colwell and Zambruskii, 1972) were picked and then purified further by repeated subculture and staining.

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

Inoculum development and viability test

Colonies were transferred from stock cultures into 20ml of Winogradskii broth and incubated at 30±2°C with shaking (100 rpm) for 36h for maximum biomass yield. Cells were suspended in sterile physiological saline, shaken in vortex mixer (Hook and Tucker Instruments, England) 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 (Okpokwasili and Odokuma, 1996).

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 incubated at 30 ± 2°C for 1h with shaking. Nitrite-N was not detected after incubation in the dark. This showed 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 showed that the disappearance of nitrite-N in the sample flasks (unautoclaved culture) was due to metabolic activities of the cells.

Nitrite solution

Nitrite solution was prepared by dissolving 0.25mg of sodium nitrite in 970ml of deionized water and dispensed in 97ml amounts into each of ten 250ml Erlenmeyer flasks. Into each of triplicate set of the above flasks was added the appropriate quantity of toxicant (0.001mg, 0.01mg, 0.10mg, 1.00mg and 10.00mg). Controls consisted of 250 ml Erlenmeyer flasks each containing the nitrite solution without any toxicants added. Sterilization was by membrane filteration (0.2µm pore size Acrodisc, France) as autoclaving resulted in precipitate formation.

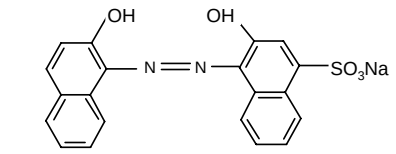
Nitrite assimilation test

Each of the triplicate set of toxicant and controls was inoculated with 3ml of bacterial inoculum (ca. 2.0 x 10^6 CFU ml^{-1}) to give final volume of 100ml per flask. Thus, the flasks contained the following concentrations of the toxicants (mg per 100ml): 0.001; 0.01; 0.10; 1.00 and 10.00. Samples (1ml) were immediately withdrawn from each flask (i.e 3ml per toxicant concentration) at zero hour and at 2h intervals and diluted 10 fold with 9ml of deionized water. Nitrite-N was then determined in the diluted sample by coupling diazotized sulphanilic acid with N-(1-naphthyl)-ethylenediamine (NED) dihydrochloride (Greenberg *et al*., 1985; Okpokwasili and Odokuma, 1996). Nitrite assimilation was determined from the calibration curve (absorbance against nitrite concentration). Results obtained as nitrite assimilation by *Nitrobacter* sp. at different concentrations of the toxicants were subjected to statistical tests using the one way ANOVA procedure (Aggarwal, 1990).

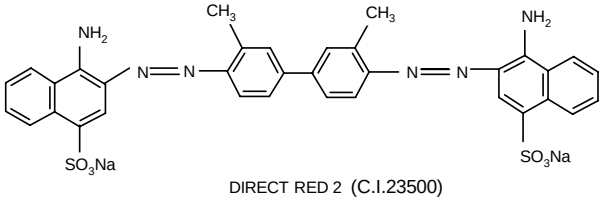
Effective concentration causing 50% inhibition (EC_{50}) was estimated by using the linear regression of the plot of percent inhibition against concentration of toxicant (Dutton, 1990). Percent inhibition was derived from the ratio of the means of nitrite assimilation (mgL^{-1}) obtained from triplicate set of flasks of control to that obtained for toxicant multiplied by 100.

RESULTS AND DISCUSSION

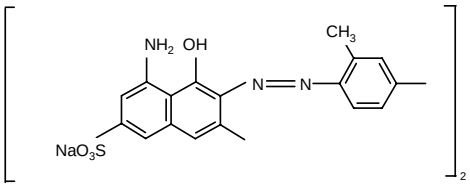
The results of the effect of the dyes (toxicants) on nitrite-N assimilation by *Nitrobacter* sp. are shown in Figures 2 - 6. In general, there was decrease in nitrite-N assimilation as the concentration of each dye increased. Data obtained for assimilation of nitrite-N at various concentrations (mgL^{-1}) of Mordant Black 17 (Fig. 2) at 8h



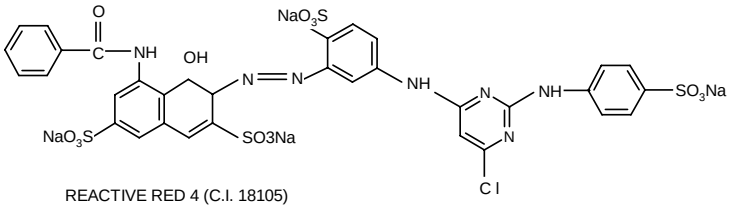
MORDANT BLACK 17 (C.I.15705)



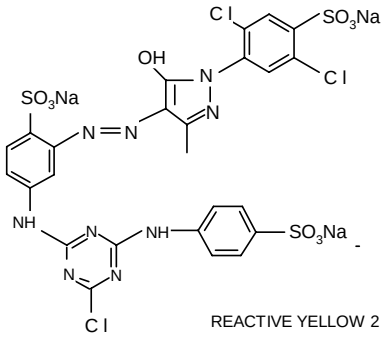
DIRECT RED 2 (C.I.23500)



DIRECT BLUE 14 (C.I. 23850)



REACTIVE RED 4 (C.I. 18105)



REACTIVE YELLOW 2 (C.I. 18972)

Fig. 1. Structures of Azo dyes used (Adapted from Aldrich catalogue, USA).

exposure time were: $0.213mgL^{-1}$ (0.01); $0.159mgL^{-1}$ (0.10); $0.104mgL^{-1}$ (1.00); $0.067mgL^{-1}$ (10.00) and $0.040mgL^{-1}$ (100.00), while values obtained for Direct Blue 14 (Fig. 4) at 8h exposure time were: $0.223mgL^{-1}$ (0.01); $0.213mgL^{-1}$ (0.10); $0.140mgL^{-1}$ (1.00); $0.066mgL^{-1}$ (10.00) and $0.035mgL^{-1}$ (100.00).

There was increase in nitrite-N assimilation with increase in exposure time at concentrations tested. Nitrite assimilation decreased significantly with increase in toxicant concentration. Statistical test using ANOVA showed that there were significant differences (p = 0.95) between nitrite assimilation values at same exposure time for various concentrations of the toxicants. Nitrite-N was not detected in the controls at 8h exposure time (Figs. 2 - 6). However, slight stimulatory activities were observed at exposure time of 2h for Reactive Red 4 (Fig. 5) and at exposure times of 2, 4 and 6h for

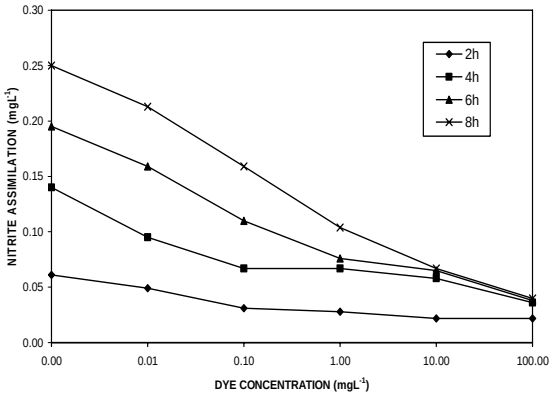


Fig. 2 Effect of various concentrations of Mordant Black 17 on nitrite assimilation by *Nitrobacter* sp.

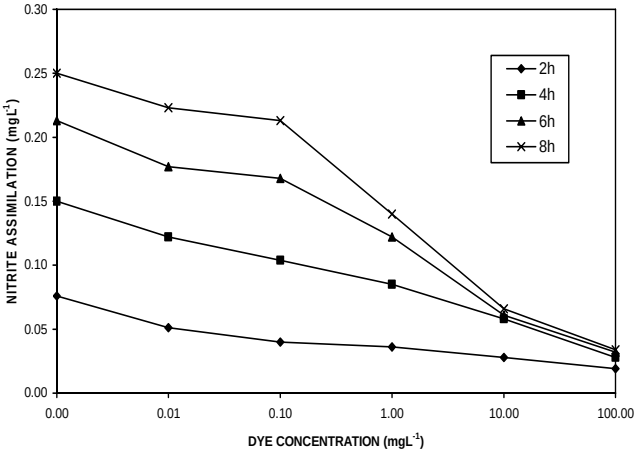


Fig. 3 Effect of various concentrations of Direct Red 2 on nitrite assimilation by *Nitrobacter* sp.

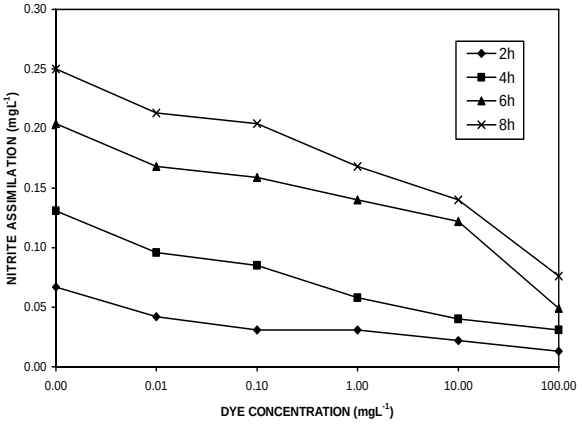


Fig. 4 Effect of various concentrations of Direct Blue 14 on nitrite assimilation by *Nitrobacter* sp.

Reactive Yellow 2 (Fig. 6). This may be attributed to hyperactivity (increase in metabolic activity of cells under stress to cope with toxic effect of toxicants at low concentrations and short exposure times). Stimulatory effects of some toxicants have been reported by previous workers [cadmium and zinc toxicity on *Nitrobacter* (Wang, 1984) and increased respiration of methanogenic culture on exposure to nickel (Speece *et al*., 1983)].

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

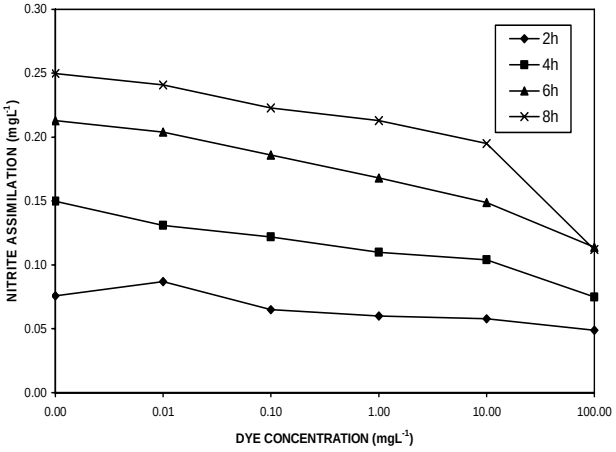


Fig. 5 Effect of various concentrations of Reactive Red 4 on nitrite assimilation by *Nitrobacter* sp.

Table 1 shows the Median Effective Concentration (EC_{50}) at various exposure times. Except for Reactive Red 4 and Mordant Black 17 , there was increase in EC_{50} with increase in exposure time. Based on EC_{50} values, the order of toxicity was: Mordant Black 17> Direct Red 2 > Direct Blue 14 > Reactive Yellow 2 > Reactive Red 4.

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 17	0.965	0.375	0.569	0.709
Direct Red 2	0.379	0.648	1.705	2.078
Direct Blue 14	0.141	0.631	8.53	11.00
Reactive Red 4	6455.5	636.03	456.76	323.97
Reactive Yellow 2	23.41	56.42	85.78	116.01

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

The increase in EC_{50} with time may be attributed to acclimation and/or detoxification which have earlier been demonstrated against *Nitrobacter* by Wang (1984).

The varying degree of toxicity exerted by the dyes may be attributed to differences in molecular weight and/or impurities in the dyes. Higher toxic effects were exerted by Mordant Black 17 and Direct Red 2 with molecular weights of 416.38 and 724.73 respec – tively compared to the other dyes with higher molecular weights of 960.32 for Direct Blue 14, 872.97 for Reactive Yellow 2 and 995.23 for Reactive Red 4.

The rate–limiting step for inhibition of cellular enzymes is the uptake rate across the cellular membrane (Klassen and Eaton, 1991). The uptake rate of lower molecular weight dyes was faster than that of higher molecular weight dyes. This may explain the higher toxicity of

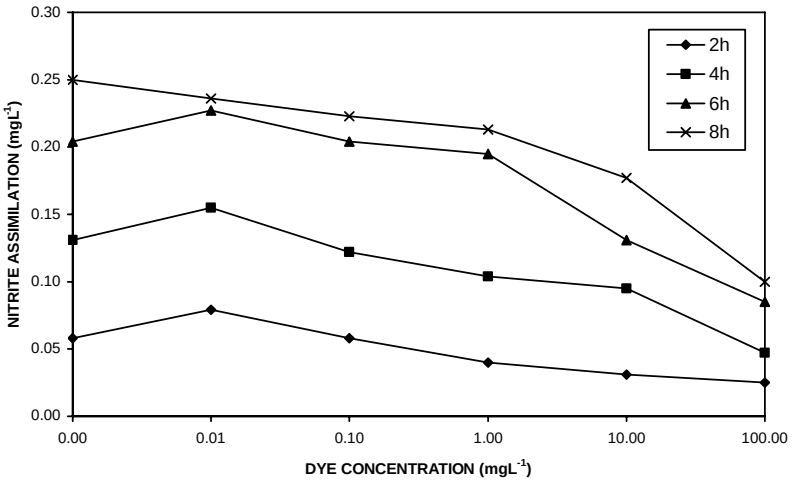


Fig. 6 Effect of various concentrations of Reactive Yellow 2 on nitrite assimilation by *Nitrobacter* sp.

Mordant Black 17 and Direct Red 2 being of lower molecular weights. Commercial azo dyes contain impurities such as aromatic amines which are intermediates in the manufacturing process of the dyes (NIOSH, 1980; Dawson, 1981; Brown and DeVito, 1993; Rafii and Cerniglia, 1995; Young and Yu, 1997; Houk *et al.,* 1999).

Aromatic amines have been reported to be toxic to various organisms [fish (Anliker *et al*., 1988); crustaceans and juvenile fish (ETAD, 1997)]. The contribution, if any, of the impurities to toxicity of the tested dyes was not investigated because the quantity of the amines in commercial azo dyes is propriety information. In addition, mordant dyes contain metallic ions (copper, zinc, chromium, aluminium) as mordants during the dyeing process (George, 1984). Shlegel (1992) reported that these metallic ions inhibited the activity of various enzymes even at very low concentrations (oligodynamic effect). Zinc and copper inhibited nitrite-N utilization by *Nitrobacter* (Wang, 1984). Heavy metals were reported to inhibit nitrification in textile treatment facilities (Hu *et al.*, 2001). The higher toxic effect of Mordant Black 17 compared to the other dyes may also be attributed to the presence of these metallic ions (Shlegel, 1992).

However, with Mordant Black 17 and Direct Red 2, the increase in nitrite-N assimilation at concentrations of $10mgL^{-1}$ and $100mgL^{-1}$ at exposure times of 4, 6, and 8h was lower compared to 2h exposure time. For example, at $10mgL^{-1}$ the difference between 4 and 6h was $0.007mgL^{-1}$ and between 6 and 8h, the value was $0.002mgL^{-1}$ compared to $0.048mgL^{-1}$ obtained between 2 and 4h exposure time. The same trend was obtained at $100mgL^{-1}$. With Direct Red 2 at $10mgL^{-1}$, the difference between exposure times of 4 and 6h was $0.003mgL^{-1}$ compared to $0.030mgL^{-1}$ between 2 and 4h exposure time. With increase in concentration and exposure time, the levels of Mordant Black 17 and Direct Red 2 with lower molecular weights and faster uptake rates attained their threshold levels at lower exposure times which resulted in decreased rate of nitrite-N assimilation.

The mechanism of azo dye inhibition of nitrite-N assimilation by *Nitrobacter* is not clearly understood. One possible explanation is competitive inhibition of the catalytic site of enzyme nitrase by the toxicants. As the intracellular concentration of the toxicants increased and competed with the substrate for the catalytic site of enzyme (nitrase), the rate of nitrite-N assimilation will decrease.

This study has shown the potentially toxic effect of the tested dyes on assimilation of nitrite-N by *Nitrobacter.* Inhibition of nitrite-N assimilation will have adverse ecological consequences on the nitrogen cycle and productivity of the ecosystem. Other studies are in progress to show the potential toxicity of other azo and non-azo dyes on *Nitrosomonas, Nitrobacter* and shrimps. It is hoped that the data accumulated will assist in formulating guidelines on discharge of dye wastewater into the environment.

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