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Tolerance of *Nitrobacter* to Toxicity of Some Nigerian Crude Oils

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Crude oil spillage in aquatic systems affects thousands of aquatic species including bacteria (Buikema Jr. et al. 1982). Some of the crude oil components are rapidly evaporated or biologically degraded (Atlas, 1981). Other components continue to remain for several months and perhaps several years (Sentsova 1979). Some of these components may be toxic to microorganisms (Gusev et al. 1982a, 1982b), while some may stimulate microbial activity especially at low concentrations (Gusev et al. 1981: Wang 1984).

The use of bacteria as bioassay organisms is now gaining wide acceptance (Williamson and Johnson 1981; Wang 1984; Vandermeulen et al. 1985; Vandermeulen and Lee 1986; Giesy et al. 1988; Dutton et al. 1990). It offers a number of advantages such as ease of handling, economy of space, short life cycles and low cost (Jardim et al. 1990). Their uses in bioassays are based on cell lysis, mutagenic properties (Vandermeulen et al. 1985) and the inhibition of physiological processes such as respiration(Wang 1984; Jardim et al. 1990).

Recently, a number of workers have proposed the use of Nitrobacter as a test organism (William and Johnson 1981; Wang 1984). The organism has a number of advantages in toxicity testing: obligate autotrophy, it's sensitivity to various toxicants (Stanier et al. 1980) and its predominance in wastewater environments are some of them (Alexander 1977). Of recent, the inhibition of bacterial enzyme biosynthesis have been suggested in bacterial assays (Dutton et al. 1990). The objective of this study was to determine the effects of six Nigerian crude oils on the cell reproduction rate (LC, lethal concentration), cellular respiration (EC, effective concentration) and biosynthesis of enzyme responsible for nitrite oxidation (IC, inhibition concentration) in Nitrobacter. In addition, the goal was to identify which of these was the most sensitive to crude oil and which may thus be used for detecting the toxicity of these chemicals.

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MATERIALS AND METHODS

Toxicity assays were conducted with Nitrobacter sp. isolated from the New Calabar Riverwater. Winogradsky medium was used for isolation. Its composition was K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.3g; NaCl, 0.3g; $MnSO_4.H_2O$, 0.02g; $FeSO_4.6H_2O$, 0.02g; $NaNO_2$, 0.05g; $ZnCl_2$, trace amounts; agar, 0.5g; deionized water, 200 ml (Colwell and Zambruski 1972). Isolates that were light grey, flat, mucoid, Gram negative, pear-shaped, and aerobic were selected according to the scheme of Colwell and Zambruski (1972).

All chemicals employed were of reagent grade and were purchased from Sigma Chemical Company, St Louis, Missouri, U.S.A. Crude oils assessed for toxicity were Bonny Medium (BM), Bonny Light(BL) and Brass River (BR). Others were Ughelli Quality Control Crude (UQCC), Qua Iboe (QI) and Trans Niger Pipeline (TNP). All crude oils were provided by the Nigerian National Petroleum Corporation (NNPC), Port Harcourt, Nigeria.

A stock solution of each crude oil (1000 mg/L) was prepared by adding the equivalent of 0.1g in volume of crude oil to 50 ml of appropriate diluent. The volume was made up to 100 ml. The mixture was strongly agitated using a rotary shaker. Serial ten-fold dilutions of this stock were produced. The diluent used for LC_{50} and EC_{50} determinations was 0.25 mg/L $NaNO_2$. Distilled water was used as diluent for IC_{50} determinations.

To 200 ml of each dilution, 5.0 ml of a 250 ml broth culture of Nitrobacter sp. was added. Control contained no crude oil. Nitrite content was immediately determined (at zero hour) as in Standard Methods for the Examination of Water and Wastewater(1985). Plates containing Winogradsky medium were immediately inoculated using the spread plate technique. Nitrite determinations and spread plate inoculations of the various crude oil concentrations after 1 h, 2 h, 3 h and 4 h incubation periods were then made. All flasks were shaken using rotary shaker and incubated at room temperature. Plates were incubated at room temperature and counted 48 h later. Nitrite loss was plotted against toxicant concentration and the median effective concentration (EC_{50}) was determined. The percent survival was plotted against toxicant concentration and the median (50%) lethal concentration (LC_{50}) was determined. Control was regarded as 100% survival.

The enzyme responsible for the oxidation of nitrite to nitrate was simply referred to as "nitritase". A modified method of Dutton et al. (1990) for the biosynthesis assay of β -galactosidase, tryptophanase and α -glucosidase was used. The modification was in the indirect estimation of the enzyme by measuring the loss of its substrate (nitrite) by the coupling of diazotized sulfanilic acid with N-(1-napthyl)- ethylenediamine (NED) - dihydrochloride (Standard Methods for the Examination of Wastewater 1985). The assay had the following steps: (1) cell growth, (2) cell preparation, (3) exposure to toxicant, (4) induction of enzyme biosynthesis, and (5) indirect measurement of enzyme activity by measurement of nitrite loss.

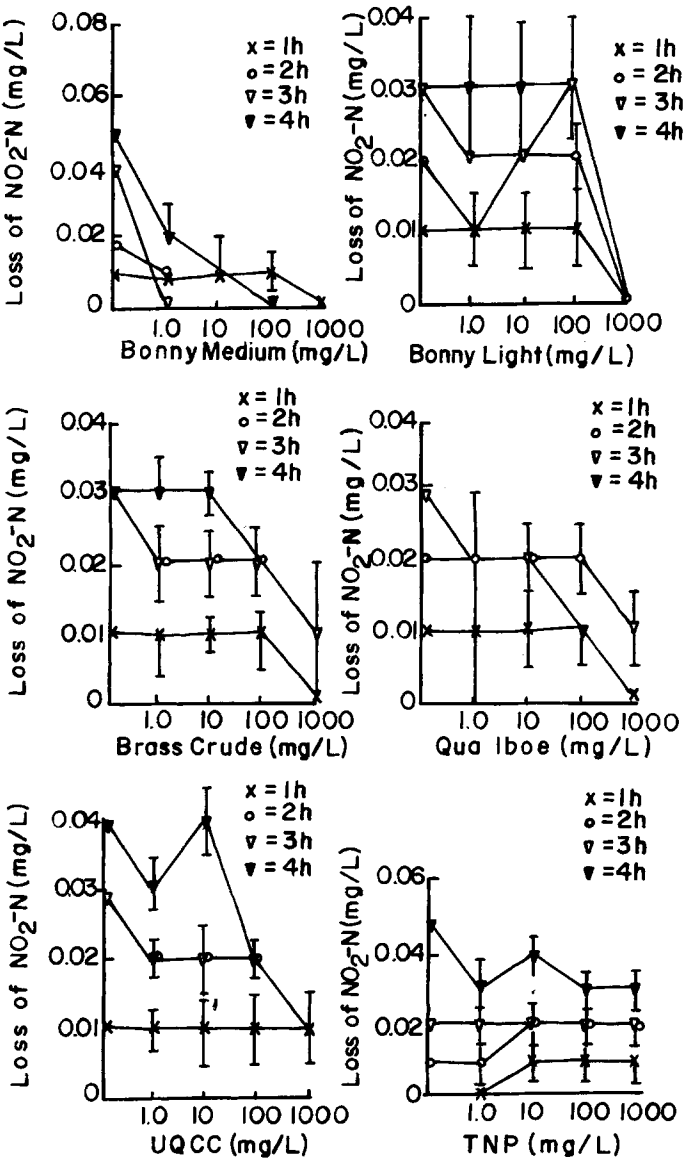

Figure 1. Effect of Six Nigerian Crude Oils on Nitrite Utilization by Nitrobacter.

Nitrobacter sp. was grown in 20 Winogradsky agar plates for 3 days. Colonies from plates were aseptically scrapped into tubes containing Winogradsky broth and diluted to A_{550} = 0.1 (Absorbance = 0.1 at 550nm). Fresh Winogradsky broth was used as blank. Biomass production of Nitrobacter is very low hence more than 20 plates may be required to achieve the above concentration. Cells were allowed to grow to A_{550} = 0.15 after which cells were crushed thrice with 0.85%(w/v) NaCl. Cells (0.1 ml of washed cell suspension at A_{550} = 0.6) were exposed to 0.7 ml toxicant and incubated for 30 min. To the reaction mixture, 5 ml of 0.25 mg/L $NaNO_2$ were added. The mixture was incubated for 60 min. Since the enzyme is intracellular, the cells were first lysed with 0.3 ml 10% SDS (w/v). This was followed by the addition of one drop each of NED-dihydrochloride and sulphanilamide reagents (Standard Methods for the Examination of Water and Wastewater 1985). The mixture was allowed to stay for 15 minutes. Absorbance of the mixture at 543 nm using a Corning colorimeter model 253 was determined. Blank consisted of all reagents used for nitrite determination minus $NaNO_2$.

All concentrations for the enzyme assays were conducted in triplicate. Preliminary range finding was carried out where possible to determine toxicant concentrations causing between 10 and 100% inhibition. Degree of sample inhibition was determined by measuring absorbance with respect to control (assigned 0% inhibition). Control contained no toxicant. The ratio of the absorbance in control to that of toxicant multiplied by 100 was taken as percent nitritase inhibition. The means of triplicate samples were obtained and used to plot a graph of percent inhibition versus concentration of toxicant. The toxicant concentration giving 50% inhibition (IC_{50}) was derived from the line of best fit of the plot. The 95% confidence limits were also calculated.

RESULTS AND DISCUSSION

The effect of six Nigerian crude oils on the nitrite utilization by Nitrobacter at 1 h, 2 h, 3 h and 4 h exposure periods are presented in Fig. 1. A decrease in the EC_{50} with exposure time was observed. The data in Table 1 clearly showed this.

Table 1. Comparison of the effect of six crudes on the 4 h LC_{50}, 4 h EC_{50} and the IC_{50} on Nitrobacter.

Crude Oil	LC_{50}	EC_{50} (mg/L)	IC_{50} (mg/L)
Bonny Medium	NT	3.0(0.3-34)	60.0(5.3-680.1)
Bonny Light	NT	10.0(0.9-113.4)	10.0(0.9-113.4)
Brass River	NT	20.0(1.8-226.7)	40.0(3.5-453.4)
Qua Iboe	NT	55.0(4.9-623)	15.0(1.3-170)
UQCC	NT	90.0(7.9-1020.2)	7500(660-85000)
TNP	NT	600(52.9-6801)	100(8.8-1133.9)

NT(Not Toxic) = 1000,000 mg/L. 95% confidence interval in parenthesis.

With respect to EC_{50} values the toxicity of crude oils decreased in the following order; BM > QI > UQCC > BL > BR > TNP. In Fig. 2,

the effect of six crude oils on the population of Nitrobacter after 1 h, 2 h, 3 h and 4 h exposure periods are presented. The data showed that the crude oils did not have any significant effect on the population. A comparison of the effect of the six crude oils on the LC_{50} and EC_{50} after a 4 h exposure period and the IC_{50} of Nitrobacter are presented in Table 1. The data indicated that LC_{50} determination was a less sensitive method for assessing Nitrobacter toxicity to crude oils. Median effective concentration (EC_{50}) and IC_{50} determinations represented more sensitive methods.

A decrease in the EC_{50} of most crude oils with exposure time indicated a decrease in energy consumption by Nitrobacter. This showed that nitrite utilization by the organism was a function of exposure time. Median effective concentrations decreased in the following order; BM > QI > UQCC > BL > TNP. The increased toxicity of most crude oils with exposure time may probably be due to increase in water solubility with time (Vandermeulen 1986; Vandermeulen and Lee 1986). Crude oil is however relatively insoluble in water. The production of water soluble fractions (WSF) between 17.5 - 26.0 µg/L by three Nigerian crude oils, Bonny medium, Bonny light and Forcados blend (FB), has been reported (Imevbore et al. 1987). With increase in exposure time there was an increase in the concentration of WSF thus increasing the effects of these water soluble toxic components on the energy consumption rate of the organism. The high EC_{50} of Bonny medium over other crude oils may be due to the high levels of toxic components within the WSF of the oil rather than a WSF concentration factor. Imevbore et al. (1987) has reported that WSF production in distilled water by three Nigerian crude oils decreased in the following order BL > BM > FB. In this study, distilled water was used to produce the various crude oil concentrations.

Trans Niger Pipeline (TNP) crude oil showed little variation in EC_{50} values with increased exposure time. This may be attributed to the high tolerance of Nitrobacter to the toxic components of their WSF. Three crude oils, BL, UQCC and TNP were slightly stimulatory to nitrite utilization at 10 mg/L.

None of the crude oils had any significant effect on the population of Nitrobacter throughout the 4 h exposure period. Thus, a decrease in energy consumption was not enough to affect the cell population suggestive of the high tolerance of Nitrobacter to Nigerian crude oil toxicity. This may be due to the inherent slow growth pattern of this organism and the short exposure time used in this study.

A comparison of the LC_{50} and EC_{50} during a 4 h exposure period and the IC_{50} value of crude oils to Nitrobacter showed that EC_{50} determinations were more sensitive for assessing crude oil toxicity to Nitrobacter. EC values measured toxicant concentrations affecting nitrite consumption while IC values measured toxicant concentrations inhibiting nitritase enzyme biosynthesis. Enzyme biosynthesis

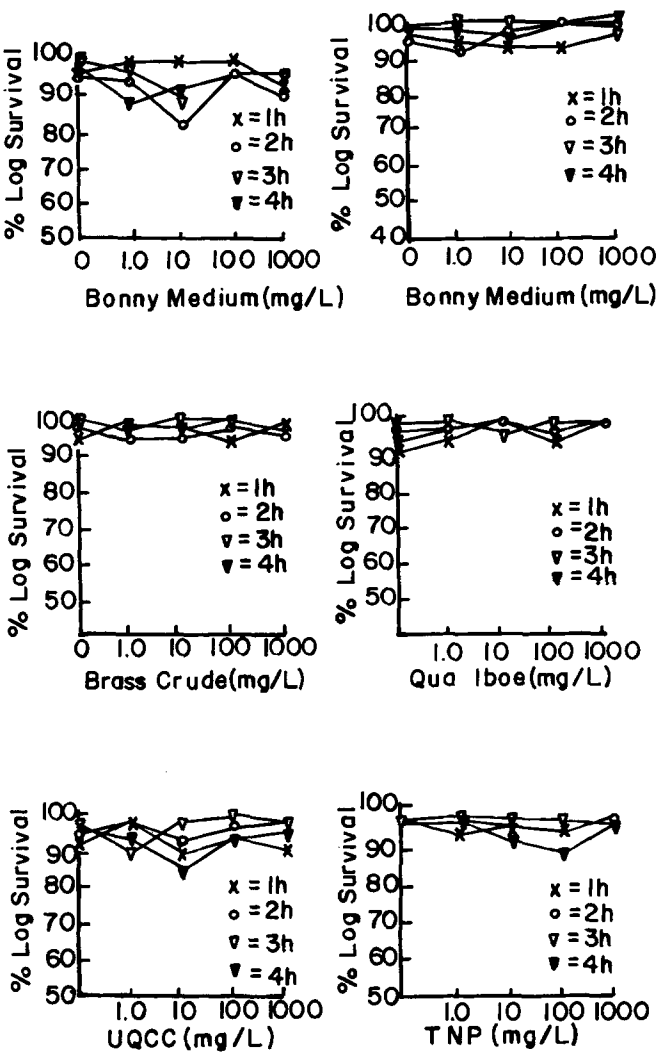

Figure 2. Effect of Six Nigerian Crude Oils on Survival of Nitrobacter.

assays were sensitive because of the permeability of the outer membrane of Nitrobacter to the WSF of crude oils. The influx of the WSF probably disrupt the activity of nitritase enzyme present within the cell. However, a disruption in the enzyme biosynthesis does not necessarily cause cell death as shown in the LC_{50} values. For IC determinations it took 2 h, for EC determinations it took about 4 h while for LC_{50} determinations, it took 12 h to achieve results.

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