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TOXICITY OF SUBSTITUTED BENZENE DERIVATIVES TO FOUR CHEMOLITHOTROPHIC BACTERIA ISOLATED FROM THE NEW CALABAR RIVER, NIGERIA

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ABSTRACT

The toxicity of benzene, hydroxylbenzene (phenol), chlorobenzene, methylbenzene (toluene) and dimethylbenzene (xylene) to four chemolithotrophic bacteria (*Nitrosomonas*, *Nitrobacter*, *Thiobacillus* and *Leptothrix* isolated from the New Calabar River water was investigated. The static method for acute toxicity assessment was employed. Mortality within a period of 5 hours exposure to toxicant was the index for assessment. Toxicity of the chemicals to the bacteria decreased in the following order: phenol > xylene > benzene > chlorobenzene > toluene for *Nitrosomonas*, chlorobenzene > phenol > benzene > toluene > xylene for *Nitrobacter*, phenol > chlorobenzene > benzene > xylene > toluene for *Thiobacillus*, while phenol > chlorobenzene > xylene > toluene > benzene was for Leptothrix. Combining the results above the toxicity of the chemicals to the test organisms decreased in the order phenol > chlorobenzene > benzene > xylene > toluene. Sensitivity of the bacteria to the test chemicals decreased in the order *Nitrosomonas* > *Leptothrix* > *Thiobacillus* > *Nitrobacter*. Toxicity of the methyl and dimethyl substituted derivatives of benzene was probably a function of the genetic make up of the bacteria. The toxicity generally decreased with increased methyl substitution in the case of *Nitrobacter* and *Thiobacillus*, but increased with increased methyl substitution in the case of Nitrosomonas and *Leptothrix*. Hydroxyl and halogenated substituted derivatives were more toxic than methyl substituted derivatives. These results indicate that wastes containing hydroxyl and chlorosubstituted derivatives of benzene may pose a greater toxicity problem to microbiota in the New Calabar River than wastes containing methyl-substituted derivatives. The nitrification stage of the nitrogen cycle will also be greatly impaired in the presence of these groups of chemicals in the river.

Key Words: Chemolithotrophic bacteria, Toxicity, Sensitivity, Benzene derivatives, New Calabar River.

INTRODUCTION

The majority of pollutants that affect the oil producing areas of the Niger Delta arise from petroleum products (Okpokwasili and Odokuma, 1990, Odokuma and Okpokwasili, 1992, Odokuma and Okpokwasili, 1993, Okpokwasili and Odokuma, 1996a,b, Odokuma and Ibor, 2002). Petroleum oil constitutes a diverse range of hydrocarbon compounds such as aliphatic, alicyclic and aromatic compounds (Atlas, 1984, 1988). The composition of each crude oil varies with its origin. Some examples of these individual compounds include cresol, benzene, resorcinol, toluene, heptane, xylene, hexane and many other hydrocarbon compounds. Substituent groups like methyl, hydroxyl nitro, amine, and halogens etc. usually attach to some of these individual components (Atlas, 1984, 1988).

Chlorinated aliphatic and aromatic hydrocarbons which are extremely toxic have become widely distributed in the environment as a result of discharges of industrial and municipal waters, urban and agricultural run off, leachate from landfill and leaking underground tanks and pipes (Phelps et al., 1990). Dinitrotoluene has been found to be mutagenic in bacterial and mammalian assay system (US Public Health Service, 1989).

The mechanisms of inhibition destruction of cellular components in microbial system by these chemicals vary (Kenneth and Johnson, 1981). They include attack on sulfhydryl groups of enzymes by halogens and alteration of the proteins to form derivatives containing halogen linked to nitrogen, disruption of cell membranes and inhibition of oxidase enzymes associated with surface membranes, etc (De Bruin, 1976). The pollutants may be toxic to microbial communities that are important in some biogeochemical cycles which include the nitrogen cycle (associated with nitrifying bacteria), sulphur cycle (associated with sulphur oxidizing bacteria) and iron cycle (associated with iron oxidizing bacteria). There are two groups of nitrifying bacteria, the ammonia oxidizers e.g., Nitrosomonas and the nitrite oxidizers e.g., Nitrobacter (Kenneth and Johnson, 1981 Wang, 1984). The sulphur oxidizing bacteria include the genus Thiobacillus which oxidizes reduced forms of sulphur to sulphates (Stanier et al., 1982). The iron oxidizing bacteria include the genus Leptothrix. They oxidize reduced forms of iron (Colwell and Zambruski, 1972, Krieg and Holt, 1994, APHA, 1998). The four organisms are chemolithotrophs (Stanier et al., 1982).

The purpose of this study was to employ these four bacterial genera as bioassay tools to evaluate the effect of methyl, chlorine and hydroxyl radical substitutions in the aromatic ring on some bacterial genera, including their

sensitivity of the organisms to these chemicals, and the toxic effects of the chemicals to these organisms.

MATERIALS & METHODS

WATER SAMPLING AND MICROBIOLOGICAL ANALYSIS

The New Calabar River is located in the Niger Delta where water is brackish due to marine water influx during tidal cycles. Surface water samples were collected from the river about 1km southwest of the University of Port Harcourt with 10ml sterile plastic containers and were analysed in the laboratory as soon as possible. The methods used for the isolation of bacteria (*Nitrosomonas, Nitrobacter Thiobacillus* and *Leptothrix*) from the river water samples were adopted from Colwell and Zambruski (1972). *Nitrosomonas* was isolated using Winogradsky medium for nitrification phase 1, *Thiobacillus* was isolated using Rodina medium, while *Leptothrix* was isolated using the Winogradsky medium for *Leptothrix* (Colwell and Zambruski, 1972). *Nitrobacter* was isolated using Winogradsky medium phase 2 as modified by Okpokwasili and Odokuma (1994). These media were autoclaved at 1210C for 15 minutes and aseptically transferred to sterile Petri dishes after cooling to about 450C. The Petri dishes were then inoculated with the river water and incubated aerobically for 4 days at room temperature (28 20C). Further identification and characterization of pure cultures of these bacteria were undertaken using the criteria of Krieg and Holt (1994). The media used for the isolation of the test organisms also served as diluent (broth media) for producing the various toxicant concentrations.

STANDARD INOCULA

Discrete colonies from each of the different culture media were subcultured into fresh media. These were then transferred into slants and stored at 40C. These slant cultures served as stock cultures. The standard inocula were prepared from the stock cultures. A loopful of the stock culture was transferred into 10ml of peptone water and incubated for 24h at room temperature (28 20C). After incubation serial dilutions of the culture were prepared to achieve 10, 102 and 103 dilutions. Appropriate medium was inoculated with these dilutions by the spread plate technique (APHA, 1998) and incubated for 48h at room temperature (2820C). The dilutions that produced colony counts of between 250 to 300 cfu/ml were chosen as the standard inocula.

TOXICANTS

The toxicants used in this study were benzene, methylbenzene (toluene), dimethyl benzene (xylene), chlorobenzene and hydroxylbenzene (phenol). These chemicals including 95% ethanol were obtained from Chadwell Health Essex England. The toxicants were first dissolved in 95% ethanol. A range of different toxicant concentrations (1000; 100.10, 1.0 and 0.1mg/l) were prepared and to the test organisms exposed to these concentrations to determine the minimum and maximum lethal concentrations of the toxicants. From this range finding test a concentration range of between 50mg/l and 1.56mg/l was observed as ideal for the toxicity test. With this range the organisms were exposed to graded concentrations (50, 25, 12.5, 3.125, 1.56 and 0mg/l, of the toxicants. These concentrations were produced as follows. The commercial concentration of benzene is 0.875g/ml. This was diluted to produce 50mg/l by mixing 0.57ml of the commercial chemical with 9.43ml of the appropriate broth medium with some 95% ethanol. This dilution produced a concentration of 50mg/l of benzene. To achieve 25mg/l of benzene 5ml of the appropriate broth medium was mixed with 5ml of the 50mg/ of benzene.

TABLE 1 CHEMICAL (TOXICANTS) USED IN THE TOXICITY TEST.

Chemical Name	Trade Name	State	Commercial Concentration
Benzene	Benzene	Liquid	0.875-0.878g/ml
Methylbenzene	Toluene	Liquid	0.863-0.866g/ml
Dimethylbenzene	Xylene	Liquid	0.860-0.866g/ml
Chlorobenzene	Chlorobenzene	Liquid	1.105-1.107g/ml
Hydroxylbenzene	Phenol	Solid crystal	

To achieve 12.5mg/l of benzene 5ml of 25mg/l of benzene was mixed with 5ml of broth medium. This procedure was used until a concentration of 1.56mg/l was obtained. The procedures for toluene and xylene were similar because their commercial concentrations were similar, 0.863 and 0.866g/ml respectively. Toluene or xylene (0.58ml) was mixed with 9.42ml of broth medium with some ethanol (95%) to achieve 50mg/l the toxicant (Toluene or xylene). The procedure was repeated as for benzene to achieve other concentration i.e., 25, 12.5, 6.25, 3.125, and 0mg/l respectively. The commercial solution of chlorobenzene has a concentration of 1.105 to 1.107g/ml. To achieve 50mg/l of chlorobenzene, 0.45ml of the commercial grade was mixed with 9.55ml of broth medium with some 95% ethanol. To achieve lower concentration a similar process as in benzene was repeated. In the case of phenol, which is in crystal form, 50mg of the crystals was dissolved in one litre of broth medium and some ethanol to produce a concentration of 50mg/l. From this concentration lower concentrations were produced as in benzene.

The standard inoculum (0.02ml) of the test organism was transferred into these toxicant concentrations and incubated for 1,2,3, 4 and 5h at room temperature 28 20C. After each of

these exposure times these cultures were inoculated into appropriate solid media by the spread plate technique (APHA 1998) and incubated for 48h at room temperature (28 20C). Plate counts were later performed. The count of bacterial cells obtained for each concentration of toxicant for a given exposure time was converted to percentage by dividing cell count of each concentration of a given toxicant by cell count of control (plate containing organisms that were not exposed to toxicant 0mg/l) and multiplied by 100. A plot of percentage cell count versus concentration of toxicants was made. The median lethal concentration (LC50) of toxicant for the various exposure times were determined using regression analysis (Finney, 1978). The analysis of variance (ANOVA) method at 95% probability level and the least significance test were used to determine which toxicant was most toxic and which organism was most sensitive to the toxicants.

RESULTS AND DISCUSSION

The median lethal concentration of benzene and its substituted derivatives to the four organisms are presented in Tables 2 to 5. During a 5h exposure to the toxicants the LC50 decreased with time. *Buikema et al.* (1982) have shown that sensitivity of test organisms to toxicity of test chemicals was a function of contact time and concentration.

Table 2: Median lethal concentration (LC_{50}) of toxicants to *Nitrosomonas*

Toxicants	1h	2h	3h	4h	5h
Benzene	9.5 ±0.5	5 ±0.5	1.5 ±0.1	0	0
Toluene	13 ±1.5	10.5 ±2.0	0	1 ±0.5	0
Xylene	9 ±2.5	7.0 ±1.5	0	0	0
Chlorobenzene	8.5 ±1.4	6.5 ±1.5	4 ±1.0	3 ±1.5	0
Phenol	7.5 ±1.5	6.5 ±1.0	0	0	0

Table 3: Median lethal concentration (LC_{50}) of toxicants to *Nitrobacter.*

Toxicants	1h	2h	3h	4h	5h
Benzene	20.5 ±2.0	19.5 ±1.5	21.5 ±2.5	20.5 ±2.5	16 ±2.5
Toluene	32.5 ±3.5	17.5 ±1.5	61 ±10.1	55 ±1.0	13 ±2.4
Xylene	26.5 ±5.0	21.5 ±2.0	44 ±11.2	13 ±5.5	31.5 ±5.0
Chlorobenzene	17.5 ±2.0	10 ±1.5	7.0 ±2.0	0	0
Phenol	16 ±2.5	11.5 ±2.0	4 ±1.5	5 ±1.5	0

Table 4: Median lethal Concentration (LC_{50}) of toxicants on *Thiobacillus*

Toxicants	1h	2h	3h	4h	5h
Benzene	34 ±5.0	17.5 ±1.0	8.0 ±1.5	0	0
Toluene	40 ±6.5	34 ±5.0	8.5 ±2.0	4±1.5	0
Xylene	35 ±4.0	25 ±6.0	10 ±2.5	3±1.0	0
Chlorobenzene	23.5 ±3.5	19.5 ±3.0	8.0 ±3.0	2.0±1.5	0
Phenol	17.5 ±3.0	9.0 ±3.0	6.10 ±2.5	4.0±1.0	0

Table 5: Median lethal concentration (LC_{50}) of toxicants on *Leptothrix*

Toxicants	1h	2h	3h	4h	5h
Benzene	26 ±3.5	18 ±2.5	16.0 ±3.0	4.0 ±0.5	5.0 ±1.5
Toluene	40 ±5.0	2.5 ±0.5	1.0 ±0.5	0	0
Xylene	12.5 ±2.5	9.5 ±2.4	7.5 ±1.0	6.5 ±0.5	5.0 ±1.5
Chlorobenzene	21.5 ±1.5	8.0 ±2.0	2.0 ±0.5	0	0
Phenol	6.5 ±0.5	11.6 ±0.5	0	0	0

At some exposure periods (3h) it was observed that the LC50 of toluene and xylene increased when it was expected to decrease (Table 3). These results indicated that certain low concentrations of toxicant were inhibitory while higher concentrations were stimulatory. These observations are reflected in the increase in the LC50, during exposure of Nitrobacter to benzene and toluene at 3h and 4h exposure period, and xylene at 3h and 5h exposure period. Similar observations have been made by Okpokwasili and Odokuma (1994, 1996a,b). They observed that certain low concentrations of crude oil, hydrocarbon fuels and drilling fluids were stimulatory while higher concentrations were inhibitory to *Nitrobacter.*

The analysis of variance at 95% probability level showed that there was a significant difference between the toxicity of the test chemicals to the test organisms. The toxicity of the chemicals decreased in the following order:

Nitrosomonas

Phenol > xylene >benzene > chlorobenzene> toluene

Nitrobacter

Chlorobenzene > phenol > benzene > toluene >xylene.

Thiobacillus

Phenol > chlorobenzene > benzene > xylene > toluene

Leptothrix

Phenol > chlorobenzene > xylene > toluene > benzene

Combining the results above the toxicity of the chemicals to the test organisms decreased in the following order:

Phenol > chlorobenzene > benzene > xylene > toluene.

Sensitivity of the organisms to the test chemicals decreased in the order:

Nitrosomonas > Leptothrix > Thiobacillus >Nitrobacter.

Phenol was generally the most toxic chemical probably as a result of hydroxyl group substitution. Phenol is known to be a protoplasmic poison and was the first antiseptic to be employed by Joseph Lister (1827-1912) (Stanier et al., 1982). The substitution of the hydroxyl radical on benzene confers a higher toxic potential to phenol. This may be due to the increased water solubility of the hydroxyl, a substituted derivative of benzene. Unsubstituted phenols display a high degree of surface toxicity and act by disrupting cell

membrane and inhibiting oxidases associated with surface membranes (De Bruin, 1976). The toxicity of chlorobenzene may be due to chlorine radical substitution of benzene nucleus. Chlorobenzene exhibits differential accumulation of bacterial cell membrane (Nishino et al., 1992).

The analysis of variance at 95% probability level showed that there was a significant difference between the toxicity patterns exhibited by the bacteria for the hydroxyl substituted benzene derivatives. The results appeared as follows:

Nitrosomonas: xylene > benzene > toluene
Nitrobacter: benzene > toluene > xylene
Thiobacillus: benzene > xylene > toluene
Leptothrix: xylene > toluene > benzene.

Xylene was more toxic to Nitrosomonas than benzene probably because xylene has two methyl substitutions in the benzene aromatic nucleus (increased methyl substitution in the aromatic ring) leading to increased toxicity (Pettibone and Cooney, 1988). Benzene was more toxic to Nitrosomonas than toluene though toluene has one methyl substitution. Patrick et al., (1991) have shown that toluene is less toxic than other substituted derivatives of benzene such as cresol, phenol, xylene, resorcinol and chlorobenzene. They have attributed this to the possession of the transfer plasmid responsible for toluene degradation (TOL plasmid) by bacteria. The toxicity pattern of Nitrobacter to these chemicals may be partly attributable to solubility factors. Solubility in aqueous medium was greatest for benzene and least for xylene. Benzene was more toxic to Thiobacillus than xylene. The higher solubility of benzene and the larger size of xylene resulting from increased methyl substitution (steric hindrance), may be responsible for this trend. The presence of the TOL plasmid may be responsible for the reduced toxicity of toluene even though it is more soluble than xylene and it is a smaller molecule (only one methyl substitution. The toxicity pattern for Leptothrix was consistent with increased toxicity and with increased substitution in the benzene nucleus (increased steric hindrance). Xylene with two methyl substitutions was more toxic than toluene with one methyl substitution, which was in turn, more toxic than benzene with no methyl group substitution.

The results also revealed that Nitrosomonas was the most sensitive of the bacteria while Nitrobacter was the least sensitive. The four organisms are Gram-negative rods. Thus although their cell wall morphology are similar (Krieg and Holt 1994), the difference in response of these bacteria to these chemicals may be due to genetic differences (Patrick et al., 1999).

CONCLUSION

The study has revealed that the toxicity of benzene and some of its substituted derivatives to these four chemolithotrophic bacteria was influenced by increased substitution in the aromatic ring, solubility of benzene and it's derivatives in water and the genetic make-up of the bacteria.

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