Global Journal of Environmental Sciences. ISSN 1596-6194. Vol. 2, No2, 2003. 128 - 132

TOLERANCE OF BACTERIA TO TOXICITY OF HEAVY METALS IN THE NEW CALABAR RIVER

*L. O. ODOKUMA AND *S. O. IJEOMAH

(Received 4 July 2002; Revision Accepted 10 March 2003)

ABSTRACT

The tolerance to salts of four heavy metals by pure and mixed cultures of four heavy metalresistant bacteria, *Bacillus, Proteus, Alcaligenes* and *Arthrobacter*, isolated from the New Calabar River water was investigated. Heavy metal resistant bacteria were isolated from river water using nutrient agar supplemented with 20g of salts of heavy metals per milliliter. Salts of heavy metals employed were $CuCl_2.2H_20$, $ZnS0_4.7H_20$, $CdCl_2.6H_20$ and Pb_30_4. *Bacillus* was the most tolerant to the four heavy metal salts while *Proteus* showed the highest degree of sensitivity. The 24h LC_{50} of $CuCl_2.2H_20$ and $ZnSO_4.7H_20$ to *Bacillus, Alcaligenes, Arthrobacter* and *Proteus* were 100mg/L, 0.3mg/L, 0.5mg/L, 0.3mg/L, and 1.0mg/L, 0.4mg/L, 0.07mg/L, and 0.008mg/L, respectively. The 24h LC_{50} of $CdCl_2.6H_20$ and Pb_30_4 to *Bacillus, Alcaligenes, Arthrobacter* and *Proteus* were 100mg/L, 100mg/L, 0.06mg/L, 0.07mg/L, 0.02mg/L, 0.2mg/L, 0.02mg/L and 0.07mg/L respectively. Hydrated Copper Chloride ($CuCl_2.2H_20$), $CdCl_2.6H_20$ and Pb_30_4 were significantly less toxic to these organisms at 1% probability level, compared to $ZnS0_4.7H_20$. Levels of heavy metals in river water ranged from <0.001 to 0.06mg/L for cadmium, 0.01 to 1.2mg/L for copper, 0.013 to 0.48mg/L for zinc, 0.007 to 0.44mg/L for iron. Vertical variation in the concentrations of heavy metals in the river water was significant at 1% probability level. River sediment concentrations of heavy metals were significantly greater than river water levels at 5% probability level. Dry season levels of heavy metals were significantly greater than rainy season levels at 5% probability level. Heavy metal concentrations of some industrial effluents discharged into the river ranged from 0.02 to 0.07mg/L for cadmium, 0.01 to 0.09mg/L for copper, 0.09 to 0.50mg/L for lead, 0.26 to 0.47mg/L for zinc and 0.42 to 2.31mg/L for iron. Results suggest that all the four heavy metal salts were toxic to river water micro flora. Hydrated zinc chloride ($ZnCl_2.7H_20$) was the most toxic to the bacteria while Pb_30_4, $CdCl_2.6H_20$ and $CuCl_2.2H_20$ displayed similar levels of toxicity. Results also indicated that the heavy metal content of the industrial effluents sited along the New Calabar River water may pose toxicity problems especially to the abundance and diversity of the existing river water microflora.

Keywords: Tolerance, Sensitivity, Resistance, Toxicity, Concentration.

INTRODUCTION

Anthropogenic activities have resulted in elevated concentrations of metals in many aquatic environments. These concentrations are of great interest in environmental studies because of their detrimental effects on natural environment (Pardo *et al.*, 1990, Li and Tong, 1991).

Once in the aquatic systems, metals are partitioned among the various aquatic environmental compartments (water, suspended solids, sediments and biota). Thus occurring in the aquatic system in dissolved, particulate and completed form (Pardo *et al.*, 1990). Heavy metal polluted environments have for some time now received wide attention in the industrialised regions of the world because of some well publicised health disasters. The Minamata and Itai-itai catastrophe (Hodges, 1977) resulted from bioaccumulation of mercury and cadmium in fish and mice respectively. Also, high concentrations of copper, nickel and zinc in soil are known to be phytotoxic while cadmium is zootoxic at low concentrations (Environment Canada, 1985).

Source control, acid treatment and addition of natural and synthetic polymers have been employed to remove metals from waste waters (Wheatland *et al.*, 1975; Metzner, 1977; Kiff *et al.*, 1983;). However, the technique of selective bioaccumulation of heavy metals by microbial systems offer the best possible approach for the remediation of contaminated water bodies. Microorganisms have developed several mechanisms at the cellular and molecular levels to overcome heavy metal toxicity changes in their external environment. Such changes include interference with microbial metabolism or alteration of the physicochemical environment of cells (Gadd, 1992; Gadd and White, 1993).

Heavy metal resistance in microorganisms can occur by a variety of mechanisms, including physical sequestration, exclusion and/or efflux, reduced uptake, detoxification, and synthetic binding proteins (Laddaga and Silver, 1951; Higham *et al.*, 1984.; Meissner and Faulkinham, 1984.; Mullen *et al.*, 1989).

The New Calabar River has witnessed a lot of perturbation as a result of increasing industrial activities related to exploration, refining and other allied activities along its bank. Such activities coupled with industrial effluent discharges may have led to wide scale contamination of its creeks and rivers with compounds containing heavy metals. The need to monitor the toxicity of predominant heavy metals on the existing bacterial flora of the river is therefore of significant environmental importance as these organisms play a vital role in biogeochemical cycles and the biotransformation of these metals. The abundance and diversity of these organisms may also be affected by these metals.

MATERIALS AND METHODS

Source of Bacteria:

The New Calabar River is located in the Niger Delta region within Rivers State of Nigeria. The water is brackish and

*Department of Microbiology, University of Port-Harcourt, Port-Harcourt, Nigeria.

slightly acidic and undergoes marine influx associated with tidal cycles. The river has industries located along its course.

Water Sample Collection

Water samples were obtained from three sites along the course of the river. The sites were coded 1, Upstream; 2, Midstream and 3, Downstream. River Water was obtained from ten random spots 15cm (surface water) and bulked together (Composite samples), in well-labelled sterile 100ml Jerry cans. This procedure was also performed for river water at depth 15-30cm (subsurface water) and sediment. The Eckman Grab sampler was used to obtain sediment samples. The sediment samples were placed in polyethene bags. River water and sediment samples were obtained from each of the three sites during the dry season and during the rainy season. Samples collected were transported to the laboratory and stored in the refrigerator at 4°C.

Chemical And Microbiological Analysis

Analysis was carried out within 24 - 48h of collection.

Treatment of Sediment Samples for Heavy Metal Analysis

The method used was adapted from APHA (1985). Each sample was digested with a mixture of concentrated nitric acid, perchloric acid, and sulphuric acid. One gram of sediment was oven dried at 60°C overnight to steady weight. This was treated with 10ml nitric acid, 10ml perchloric acid and 0.5m1 sulphuric acid. Cooling was done between additions in a cold-water bath. Each sample was evaporated gently on a hot plate until dense white fumes of perchloric acid appeared. The clear solution formed was then cooled and diluted to 50ml with distilled water.

Heavy Metal Analysis

The method used was adopted from APHA (1985). The heavy metals in the samples were determined using model AA320 atomic absorption spectrophotometer (Shanghai Analytical Instrument Co.).

Isolation of Heavy Metal Resistant Bacteria from the River water.

Heavy metal resistant bacteria were isolated from the river water. Weighed fractions of sediments (1g) and 1ml of water samples were introduced into tubes containing 9m1 of 0.85% normal saline. Contents were then mixed thoroughly and 0.1 ml were transferred unto Nutrient Agar plates supplemented with 20g of metals per ml. All plates were run in triplicates and incubated at 37°C for 24h.

Isolates obtained were characterised and identified by criteria present in Krieg and Holt (1994). Isolates were purified by subculturing. Pure colonies were aseptically transferred unto Tris-minimal medium (Diels, 1997) (Tris-HCl,6060mg; NaCl,4680mg; KCl,1490mg; NH_4Cl,1070mg; Na_2SO_4,430mg; $MgCl_2.6H_20$,200mg; $CaCl_2.6H_20$,30mg. B-glycerophosphate,29mg; $Fe(NH_4)$ citrate,11mg; Lactate,8000mg; $MnCl_2.4H_20$,l00mg; H_3BO_3,62g; $CuCl_2.6H_20$,190g; $CuC1_2.2H_20$,17g; $NiCl_2.6H_20$,24g; $Na_2Mg0_4.2H_20$,36g; $ZnSO_4.7H_20$,144g; Agar, 15g) to confirm resistance. Isolates showing growth on medium were transferred onto slants and stored. Prime consideration in this selection was colouration and peculiar characteristics on Nutrient agar. This was to enable the disappearance or persistence of the organism to be monitored as they were exposed to toxicants.

Toxicity Test

A standard inoculum for this test consisted of 200ml of sterile water to which a loopful of 24h, culture of each isolate chosen was aseptically introduced into it. The mixture was allowed to acclimatise for a period of 30mins. The Total Viable Count (TVC) was carried out to estimate the number of viable organisms. Individual isolates were equally prepared as above.

Different toxicant concentrations were prepared by dissolving 1g of salt of heavy metal into l litre of sterilised deionised water to give a concentration of 1000mg/L. Subsequent concentrations were obtained by ten - fold serial dilution of 1ml of stock to give 100mg/l, 10mg/l, 1mg/l, 0.1mg/l, and 0.01mg/l respectively. Salts of metals used were:- $Pb_30_4CdCl_2.6H_20$, $CuCl_2.2H_20$ and Zn $SO_4.7H_20$.

Test Procedure

- One milliliter of each standard inoculum was introduced into the different dilutions of heavy metal salts using a sterile pipette. Controls contained no salt of heavy metal, only normal saline. Nutrient agar plates were immediately inoculated by spread plate technique (APHA 1985) to give 0h period. Spread plate inoculations of mixture 0.1 ml salt dilutions containing pure or mixed cultures were carried out after 1h, 3h, 6h, 12h, 18h, and 24h incubation periods on nutrient agar plates and incubated at 37°C for 24h. Percentage survival of each isolate and/or mixed culture were plotted against toxicant concentration and the median lethal concentration (LC_{50}) was determined by regression analysis (Finney, 1978). Results from control were regarded as 100% survival.

CALCULATIONS

The toxicity of the salts of the heavy metals were evaluated by adopting the all or none test (that is setting two hypothesis alive or dead) from which the relationship between concentration and percentage effect were deduced. The concentrations were plotted against percentage survival using Probit graph. Regression analysis was then used to obtain the line of best fit from which the LC_{50} were determined for the 24h exposure period. To determine which organism were more sensitive to each salts, one way analysis of variance was performed (Finney, 1978) using their LC_{50} values.

RESULTS AND DISCUSSION

Four bacterial isolates *Bacillus, Proteus, Alcaligenes* and *Arthrobacter* were used as mixed and pure cultures to monitor the toxicity of the four heavy metals. In figure 1 the effect of $CuCl_2.2H_20$ on the percentage survival of the mixed bacterial culture after 24h exposure period is presented. Results show that concentration of 0.01, 0.1 and l000mg/L of the metal salt were more toxic to the mixed culture than concentrations between these extremes. Okpokwasili and Odokuma (1996a,b) have observed similar results when *Nitrobacter* was exposed to drilling muds and refined petroleum products (diesel, aviation kerosene, cooking kerosene and petrol).

In figure 2 the percentage survival of the mixed bacterial cultures generally decreased with increase in concentration of $ZnSO_4$.7H20. Mortality was instant on exposure of the mixed culture to 1000mg/L of $ZnSO_4.7H_20$, unlike that of $CuCl_22H_20$, which even at 1000rng/L after 24h exposure a large proportion of the organisms still survived. Figure 2 even revealed that after 24h of exposure even 10mg/L of the $ZnSO_4.7H_20$ was toxic to the mixed culture. This suggested that $ZnSO_4.7H_20$ at

Table 1: Percentages(%) of bacteria Genera isolated from the New Calabar River water during the rainy and dry seasons.

Genus	Site 1		Site 2		Site 3	
	Rainy season	Dry season	Rainy season	Dry season	Rainy season	Dry season
Bacillus	36	65	40	72	38	92
Arthrobacter	14	9	15	8	22	2
Proteus	14	16	8	-	4	-
Alcaligenes	26	6	13	18	23	6
Lactobacillus	-	-	10	-	-	-
Salmonella	-	-	2	-	-	-
Staphylococcus	10	4	22	2	13	-

Table 2: Median Lethal Concentration (24h LC_{50}) of Heavy metal salts to pure cultures of four Bacteria.

Organisms	$CuCl_2.2H_2O$	$ZnSO_4.7H_2O$	$CdCl_2.6H_2O$	Pb_3O_4
	24h LC_{50} (mg/L)			
Bacillus	100 ±5.5	1.0 ±0.2	100 ±10.0	100 ±8.5
Alcaligenes	0.3 ±0.01	0.4 ±0.03	0.06 ±0.002	0.07 ±0.001
Arthrobacter	0.5 ±0.02	0.07 ±0.001	0.02 ±0.001	0.2 ±0.001
Proteus	0.3 ±0.01	0.008 ±0.001	0.02 ±0.001	0.07 ±0.005

Result represents means and standard deviations of three replicates.

Table 3: Heavy Metal levels of the New Calabar River water during rainy season.

Site	Depth	Mean concentration of heavy metal (mg/L)				
			Cu d	C •	Pb Z n	Fe
1	0 - 15cm	< 0.001	0.03 ±0.001	0.067 ±0.004	0.007 ±0.001	29.64 ±3.0
	15 - 30cm	< 0.001	0.01 ±0.001	0.083 ±0.005	0.007 ±0.001	30.02 ±2.5
	Sediment	< 0.001	1.093 ±0.001	0.27 ±0.04	0.034 ±0.001	109.6 ±9.5
2	0 - 15cm	< 0.001	0.014 ±0.001	0.009 ±0.001	0.015 ±0.001	38.2 ±5.0
	15 - 30cm	< 0.001	0.029 ±0.001	0.013 ±0.001	0.014 ±0.001	34.69 ±3.0
	Sediment	< 0.009	1.348 ±0.001	0.313 ±0.04	0.066 ±0.005	109.6 ±10.1
3	0 - 15cm	< 0.001	0.055 ±0.002	0.074 ±0.003	0.013 ±0.001	30.01 ±2.5
	15 - 30cm	< 0.001	1.201 ±0.03	0.059 ±0.005	0.022 ±0.001	30.01 ±2.5
	Sediment	< 0.001	1.448 ±0.02	1.269 ±0.03	0.057 ±0.001	62.63 ±5.5

Result represents means and standard deviations of three replicates.

l0mg/L after 24h of exposure was very toxic to these organism thus industrial effluents containing concentrations of $ZnSO_4.7H_20$ as much as 10mg/L would definitely pose serious toxicity problems to the micro biota of such receiving systems.

In figure 3 the effect of cadmium chloride ($CdCl_2.6H_20$) on the percentage survival of the mixed bacterial culture is presented. One hundred percent mortality of the mixed bacterial culture was observed after 3,6,12,18 and 24h exposure to 100mg/L of the salt. This suggested that this salt was toxic to the mixed bacterial culture. It was probably more toxic than $ZnSO_4.7H_20$.

The effects of trilead tetraoxide (Pb_30_4) on the percentage survival of mixed bacterial cultures are presented in Figure 4. The results show a decrease in percentage survival (increase in mortality) with increase in the concentration of the metal. Concentrations of 10mg/L and 1000mg/L produced 100% mortality after 18h exposure of this mixed culture to the metal. There was a sharp increase in mortality of the mixed culture at 0.01 mg/L of Pb_30_4. This result show that increasing concentration of Pb_30_4 were generally toxic to the mixed culture. This was not however the case in figure 1 ($CuCl_2.2H_20$) and figure 3 ($CdCl_2.6H_20$) where some metal salt concentrations were stimulatory to microbial growth. Similar results have been observed by Okpokwasili and Odokuma (l 996a,b).

In table 1, the predominant heavy metal resistant bacterial genera encountered in the New Calabar River water are represented. Results indicated that *Bacillus* was the most predominant while *Lactobacillus* and *Salmonella* were the least.

The 24h median lethal (LC_{50}) of the salts of the four heavy metals to pure cultures of the test organisms are presented in table 2. *Bacillus* was the most tolerant to $CuC1_2.2H_20$ while the other organisms displayed similar levels of sensitivities to the salt. All four organisms were sensitive to $ZnSO_4.7H_20$. *Bacillus* with an LC_{50} of 1.0mg/L was more tolerant to the salt while *Proteus* with an LC_{50} of 0.008mg/L was the most sensitive. *Bacillus* was the most tolerant to $CdCl_2.6H_20$ with an LC_{50} of 100mg/L. Other organisms were more sensitive to this salt with an LC_{50} of 0.02 to 0.06 mg/L respectively. The 24h LC_{50} of *Bacillus* to Pb_30_4 was 100mg/L (table 2) indicating that this organism was more tolerant to the salt. Other organisms were more sensitive to the salt with an LC_{50} of 0.09 to 0.7mg/L. *Proteus* was the most sensitive to the salt.

These results reveal varied tolerance levels to the heavy metal salts by the different organisms. *Bacillus* was the most tolerant organism to all the metal salts. This may not be unconnected to the presence of endospores that may have shielded it from the lethal action of the heavy metals. *Bacillus* may also possess wel1-characterised genetic plasmids. (Okpokwasili *et al., 1986)* which confers resistance to the organism. The tolerance to heavy metal toxicity by pure cultures followed the trend *Bacillus>Alcaligenes>Arthrobacter>Proteus*. The toxicity of the metal salt to the pure cultures showed the following trend $ZnCl_2.7H_20$ was the most toxic while Pb_30_4, $CuCl_2.2H_20$ and $CdCl_2.H_20$ displayed similar levels of toxicities. *Bacillus* was

Table 4: Heavy Metal level of the New Calabar River water during the dry season

		Mean concentration of heavy metal (mg/L)				
Site	Depth	Cd	Cu	Zn	Pb	Fe
1	0 - 15cm	0.06 ± 0.001	0.220 ± 0.02	0.40 ± 0.01	0.230 ± 0.03	44.12 ± 2.2
	15 - 30cm	0.03 ± 0.001	0.200 ± 0.03	0.48 ± 0.02	0.230 ± 0.02	42.35 ± 2.4
	Sediment	0.03 ± 0.001	0.090 ± 0.01	0.710 ± 0.03	0.560 ± 0.01	113.5 ± 2.3
2	0 - 15cm	0.02 + 0.001	0.070 + 0.02	0.300 + 0.01	0.230 ± 0.03	40.74 ± 2.5
	15 - 30cm	0.023 ± 0.001	0.090 ± 0.01	0.220 ± 0.02	0.440 ± 0.02	45.34 ± 2.4
	Sediment	0.04 ± 0.001	0.120 ± 0.02	0.490 ± 0.01	0.560 ± 0.01	122.18 ± 2.2
3	0 - 15cm	0.04 ± 0.001	0.100 ± 0.03	0.190 ± 0.02	0.420 ± 0.01	36.68 ± 2.2
	15 - 30cm	0.007 ± 0.001	0.072 ± 0.02	0.194 ± 0.03	0.411 ± 0.02	38.26 ± 2.5
	Sediment	0.05 ± 0.001	0.180 ± 0.03	0.520 ± 0.01	0.410 ± 0.01	64.50 ± 3.4

Result represents means and standard deviations of three replicates.

Table 5: Heavy Metal levels of effluents of three industries located along the New Calabar River

		Mean concentration of heavy metal (mg/L)				
Site	Season	Cd	Cu	Zn	Pb	Fe
1	Rainy	0.07 ± 0.01	0.06 ± 0.001	0.28 ± 0.02	0.22 ± 0.01	0.42 ± 0.03
	Dry	0.06 ± 0.01	0.05 ± 0.001	0.36 ± 0.03	0.50 ± 0.02	0.86 ± 0.05
2	Rainy	0.10 ± 0.001	0.09 ± 0.001	0.42 ± 0.01	0.15 ± 0.001	1.08 ± 0.05
	Dry	0.12 ± 0.001	0.06 ± 0.001	0.54 ± 0.02	0.42 ± 0.02	1.64 ± 0.04
3	Rainy	0.02 ± 0.001	0.01 ± 0.001	0.26 ± 0.04	0.09 ± 0.002	1.94 ± 0.03
	Dry	0.03 ± 0.001	0.03 ± 0.001	0.47 ± 0.02	0.35 ± 0.01	2.31 ± 0.05

Result represents means and standard deviations of three replicates.

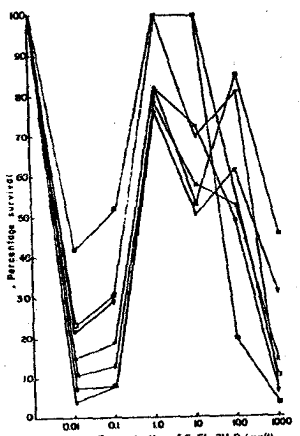

Fig 1. Effect of Copper II Chloride on the percentage survival of mixed Bacterial culture. # - # 0hr, □ - □ 1hr. V-V 3hr. ● - ● 6hr. x - x 12hr. v - v 18hr ■ - ■ 24hr.

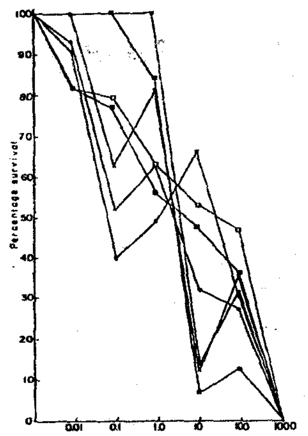

Fig 2. Effect of Zinc Sulphate on the percentage survival of mixed Bacterial culture. # - # 0hr, □ - □ 1hr. V-V 3hr. ● - ● 6hr. x - x 12hr. v - v 18hr ■ - ■ 24hr.

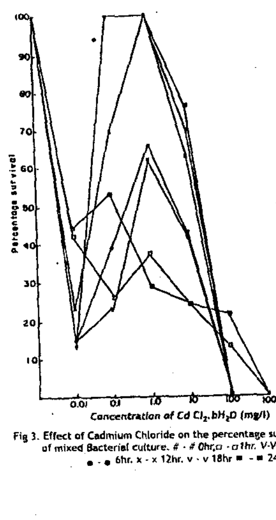

Fig 3. Effect of Cadmium Chloride on the percentage survival of mixed Bacterial culture. # - # 0hr, □ - □ 1hr. V-V 3hr. ● - ● 6hr. x - x 12hr. v - v 18hr ■ - ■ 24hr.

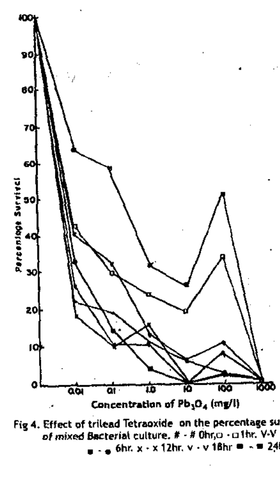

Fig 4. Effect of trilead Tetraoxide on the percentage survival of mixed Bacterial culture. # - # 0hr, □ - □ 1hr. V-V 3hr. ● - ● 6hr. x - x 12hr. v - v 18hr ■ - ■ 24hr.

the most tolerant of the four organisms. The solubility of salts may have played an important role in the amount of each metal exposed to isolates. Studies have shown that the chloride of metals is preferred to other anions in toxicity experiment because they are less toxic. (Plimmer, 1978).

Seasonal variation in the heavy metal levels of the river water (0-15cm and 15-30cm depth) and the sediment at three stations along the river are presented in tables 3 and 4. Results indicated that during the rainy season the concentrations of the heavy metals were lower than during the dry season. This may be due to dilution effect of increased water volume during the rainy season. Results however suggested that sediment levels of heavy metals were generally higher than the river water. This may be due to accumulation of the heavy metals in the sediment because of its relative immobility as compared to the river water.

In table 5 the heavy metal concentration of the effluent of three industries located along the river are presented. The effluent concentrations of most heavy metals (Pb, Cd, Zn and Cu) were similar to the dry season levels of heavy metals in the river water. The rainy season levels of the heavy metals in the river water were less than the dry season levels, thus less than the effluent levels. Iron levels of river water of both seasons were however higher than effluent levels. This study has revealed that the salts of heavy metals such as Pb, Cd, Zn and Cu arc toxic to pure and mixed bacterial cultures isolated from the river water. The study suggested that the concentration of heavy metals in the effluent could pose toxicity problems to the micro flora of the river water (Figures 1,2, 3, and 4).

Results of toxicity tests using pure cultures of the bacteria indicated that the heavy metal levels of the effluents are generally greater than the 24h LC_{50} of the metal salts especially during the dry season. This situation may affect the population and biodiversity of the river water. *Bacillus* was the most tolerant of the four test bacteria. Thus *Bacillus* may predominate in the river water, while other more sensitive genera may decrease in population. This may affect the ecological balance of such aquatic habitats.

Conclusion

The study reveals that heavy metals and their levels in effluents of industries sited along the river may pose toxicity problems to microorganisms that inhabit river water. In this study *Bacillus* was the most tolerant of the four isolates *Proteus* was the most sensitive. Hydrated Copper II chloride ($CuCl_2.2H_2O$), $CdCl_2.6H_2O$ and Pb_3O_4 displayed similar levels of toxicity while $ZnSO_4.7H_20$ was the most toxic. The microbial responses to different concentration of the heavy metal salts were varied in mixed cultures. The results reveal that the current heavy metal level of the river water may pose toxicity problems to the existing micro flora.

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