International Scholars Journals

International Journal of Plant and Animal Sciences ISSN 2167-0366 Vol. 7 (2), pp. 001-006, February, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Response of planktonic bacteria of New Calabar River to zinc stress

C. O. Nweke[1]*, J.C. Okolo[1], C. E. Nwanyanwu[1] and C. S. Alisi[2]

[1]Department of Microbiology, Federal University of Technology, P.M.B.1526, Owerri, Nigeria.
[2]Department of Biochemistry, Federal University of Technology, P.M.B. 1526, Owerri, Nigeria.

Accepted 18 November, 2018

Toxicity of Zn^{2+} on four planktonic bacteria isolated from New Calabar River water was assessed via dehydrogenase assay. Pure cultures of the bacterial strains were exposed to various Zn^{2+} concentrations (0.2 - 2.0 mM) in a nutrient broth amended with glucose and TTC. The responses of the bacterial strains to Zn^{2+} is concentration- dependent. At 0.2 mM, Zn^{2+} stimulated dehydrogenase activity in *Proteus* sp. PLK2 and *Micrococcus* sp. PLK4. In all strains, dehydrogenase activity was progressively inhibited at concentrations greater than 0.2 mM. The IC_{50} ranges from 0.236 ± 0.044 to 0.864 ± 0.138 mM. Total inhibition occurred at concentrations ranging from 1.283 ± 0.068 to 2.469 ± 0.045 mM. The order of zinc tolerance is: *Micrococcus* sp. PLK4 > *Proteus* sp. PLK2 > *Pseudomonas* sp. PLK5 > *Escherichia* sp. PLK1. The result of the *in vitro* study indicated that the bacterial strains are sensitive to Zn^{2+} stress. Therefore, Zn^{2+}contamination would pose serious threat to their metabolism in natural environments.

Key words: New Calabar River, zinc toxicity, planktonic bacteria, dehydrogenase.

INTRODUCTION

Heavy metals have great ecological significance due to their toxicity and accumulative behaviour (Purves, 1985). Unlike organic pollutants, heavy metals are not biodegradable and undergo a global eco-biological cycle (Nürnberg, 1984) in which natural waters are the major pathways. Anthropogenic activities have resulted in the increased levels of trace metals in many aquatic environments. Heavy metals discharged into estuarine and coastal waters rapidly become associated with particulates and are incorporated in bottom sediments (Hanson et al., 1993). Diagenetic processes and physical disturbances in the sediments can cause spatial translocation of these contaminants between the sediment and the water phases and disturb the activity of the planktonic microorganisms.

Microorganisms are vital for the efficient functioning of any ecosystem, thus, factors that affect their metabolism, distribution, and abundance are of great concern. Microbes respond quickly to environmental pollution and monitoring microbial responses has been recommended as an early warning indicator of ecosystem stress (Griffiths, 1983; Odum, 1985). Measurement of microbial enzyme activity is used in the assessment of ecotoxicological impacts of environmental pollutants. The most often studied groups of enzyme are oxidoreductases e.g. dehydrogenases. Dehydrogenase assay involving the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) and 2-(*p*-iodophenyl)-5-phenyltetrazoliumchloride (INT) to their formazans has been used to measure microbial activity (Gong, 1997; Mathew and Obbard, 2001) and to assess the toxicity of heavy metals (Aoyama and Nagumo, 1997; Kelly and Tate, 1998) and polyaromatic hydrocarbons (Klimkowicz-Pawlas and Maliszewska-Kordybach, 2003; Maliszewska-Kordybach and Smreczak, 2003).

The organic and inorganic pollution of New Calabar River has been attributed to the effluent discharges from industries sited along its bank (Okpokwasili and Odokuma, 1993; Odokuma and Okpokwasili, 1997; Odokuma and Ijeomah, 2003a). The heavy metal content, seasonal variations in the population of heavy metal resistant bacteria as well as toxicity and accumulation of heavy metals by bacteria isolated from the New Calabar River have been reported (Horsfall and Spiff, 2002;

*Corresponding author: Email: xrisokey@yahoo.com.

Odokuma and Abah, 2003; Odokuma and Ijeomah, 2003a,b). However, these studies did not consider the inhibition of dehydrogenase enzyme activity in these bacteria. This study was aimed at assessing the *in vitro* effects of zinc on the dehydrogenase activity of bacterial species isolated from New Calabar River water.

MATERIALS AND METHODS

Sample collection and analysis

The New Calabar River is a short tidal coastal river of about 150-200 km in length and flow through Port Harcourt, Rivers State in the Niger delta region of Nigeria. The water is brackish and impacted by effluent discharges from industries sited along its bank. Water samples were collected midstream along the course of the river at Choba from a depth of 30 cm. The sampling site has been described by Odokuma and Ijeomah (2003b). The samples were collected in 1litre sterile glass bottles. Samples were stored in a cooler and taken to laboratory. All samples were analyzed within 6 h of collection. The pH and zinc content of the sample were determined using pH meter (Jenway 3015) and atomic absorption spectrophotometer (Perkins Elmer 3110), respectively.

Isolation of bacterial strains and culture conditions

Aerobic heterotrophic bacteria in the samples were isolated and purified on nutrient agar plates. Purified cultures were characterized biochemically using standard microbiological methods and identified to the generic level following the schemes of Holt et al. (1994).

The bacterial strains were grown to mid exponential phase in nutrient broth (Lab M) on a rotary incubator (150 rpm) at room temperature (28 ± 2°C) and the cells harvested by centrifugation at 1000 g for 10 min. Harvested cells were washed twice in deionized distilled water to avoid any nutrient carryover. Washed cells were re-suspended in deionized distilled water and the turbidity adjusted to give an optical density of 0.85 at 420 nm. The cell suspensions were used as inoculum in the dehydrogenase activity assay. The dry weight of cells was determined by drying a 10 ml aliquot of cell suspensions (contained in pre-weighted crucibles) to constant weight in an oven at 110°C.

Dehydrogenase assay

Dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride as the artificial electron acceptor, which is reduced to the red-coloured triphenylformazan (TPF). The assay was done in 3-ml volume of nutrient broth- glucose- TTC medium supplemented with varying concentrations of Zn^{2+} as zinc sulphate in separate screw-capped test tubes. Portions (0.3 ml) of the bacterial suspensions were inoculated into triplicate glass tubes containing 2.5 ml of phthalate-buffered (pH 6) nutrient broth-glucose medium amended with Zn^{2+} and preincubated on a rotary incubator (150 rpm) at room temperature (28 ± 2°C) for 30 min. Thereafter, 0.2 ml of 0.4% (w/v) TTC in deionized distilled water was added to each tube to obtain final Zn^{2+} concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5 and 2.0 mM in different test tubes. The final concentrations of nutrient broth, glucose and TTC in the medium were 2, 2 and 0.267 mg/ml, respectively. The controls consisted of the isolates and the media without Zn^{2+}. The reaction mixtures were further incubated under static conditions at room temperature (28 ± 2°C) for 4 h. The TPF produced was extracted in 4 ml of amyl alcohol and determined spectrophotometrically at 445 nm (λmax). The amount of formazan produced was determined from a standard dose-response curve [0-200 mg/l TPF (Sigma) in amyl alcohol; R^2 = 0.9955]. Dehydrogenase activity was expressed as milligrams of TPF formed per mg dry weight of cell biomass per hour.

Zinc inhibition of dehydrogenase activity was calculated relative to the control. Inhibition data (percent inhibition) were linearized against the concentrations of toxicant using gamma parameter ( ) as shown in the equation below (Kim et al., 1994). The IC_{50}, which is an inhibitory concentration of toxicant required to reduce 50% of the dehydrogenase activity, was determined. The total inhibition concentrations were estimated from the linear regressions of log transformation plots of the dose-response data.

$$= \frac{\%\text{Inhibition}}{100 - \%\text{Inhibition}}$$

Statistical analysis

Data generated from this study were subjected to multiple factor analysis of variance (2-Way ANOVA).

Table 1. Dehydrogenase activities in the control tests.

Strain	Dehydrogenase activity[a] (mg Formazan/mg cell dry wt/h)
Escherichia sp. PLK1	0.935 ± 0.062
Proteus sp. PLK2	0.161 ± 0.050
Micrococcus sp. PLK4	0.033 ± 0.004
Pseudomonas sp. PLK5	0.256 ± 0.024

[a]Data represent mean ± standard deviation of triplicate tests.

RESULTS AND DISCUSSION

The zinc content and pH of the New Calabar River water were 5 mg/l (≈76.48 μM) and 6.4 respectively. This level of zinc is much higher than the previously reported levels of 0.01 to 0.71 mg/l (Odokuma and Abah, 2003; Odokuma and Ijeomah, 2003a,b), indicating that zinc was accumulating in the New Calabar River over time. Typical background level of zinc in freshwater and seawater systems are 0.30 and 0.153 μM respectively (Goldman and Horne, 1983; Leppard, 1981; Bidwell and Spotte, 1985). It is therefore obvious that the concentration of zinc in the river was elevated.

Four bacterial strains comprising three Gram negative (*Escherichia* sp. PLK1, *Proteus* sp. PLK2, and *Pseudomonas* sp. PLK5) and one Gram positive (*Micrococcus* sp. PLK4) organisms were isolated. Results obtained from the control samples showed that these organisms were able to reduce TTC to the red formazan at variable rates and extent (Table 1). The Gram negative bacteria had higher rates of dehydrogenase activity than the Gram positive

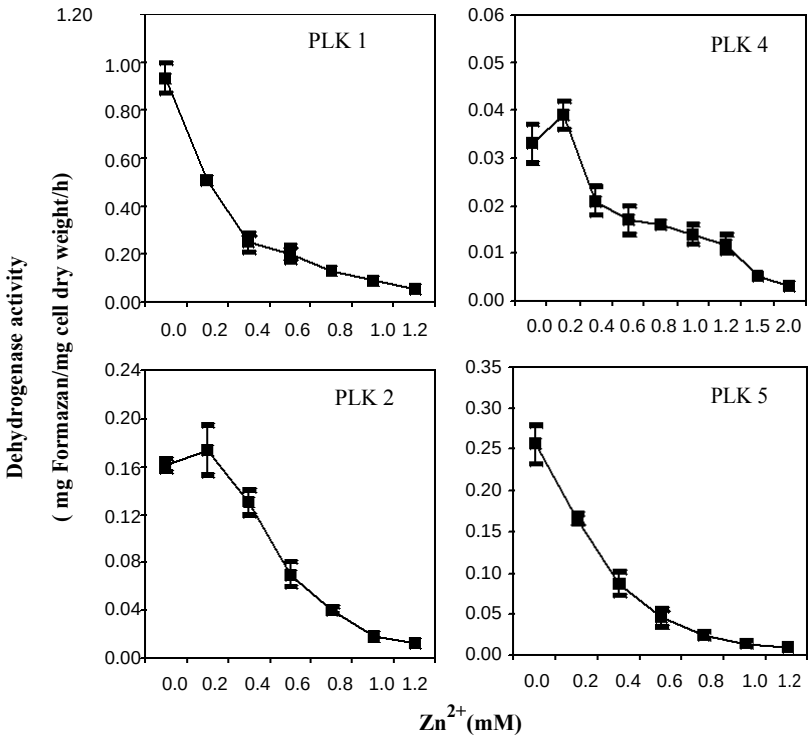

Figure 1. TTC reduction activity in response to various concentrations of zinc by *Escherichia* sp. PLK 1, *Proteus* sp. PLK 2, *Micrococcus* sp. PLK 4 and *Pseudomonas* sp. PLK 5. Means ± Standard deviation (n=3) are indicated by bars. Some standard deviations are within data points.

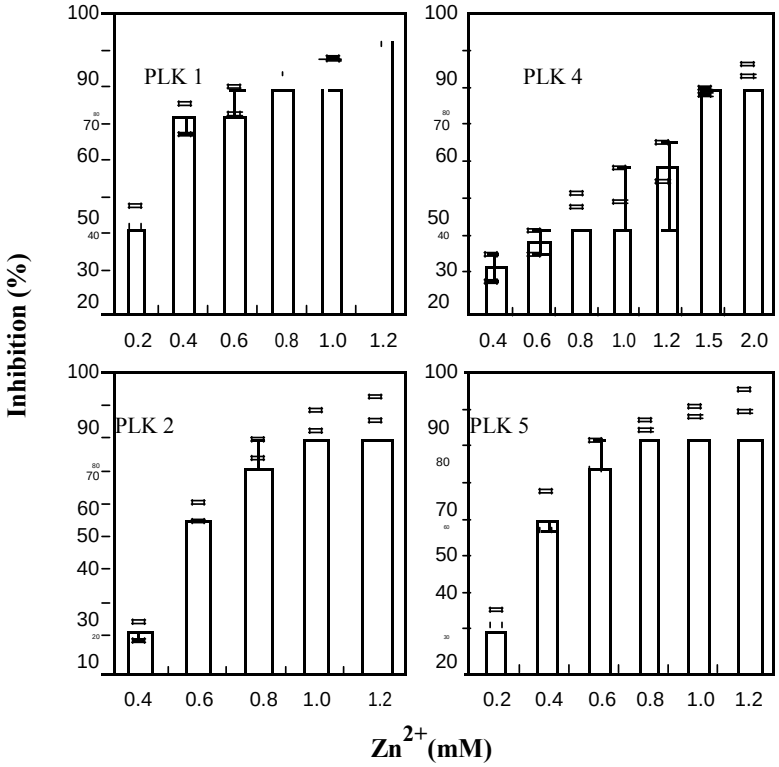

Figure 2. Zinc inhibition of dehydrogenase activity in *Escherichia* sp. PLK 1, *Proteus* sp. PLK 2, *Micrococcus* sp. PLK 4 and *Pseudomonas* sp. PLK 5. Means ± Standard deviation (n=3) are indicated by bars.

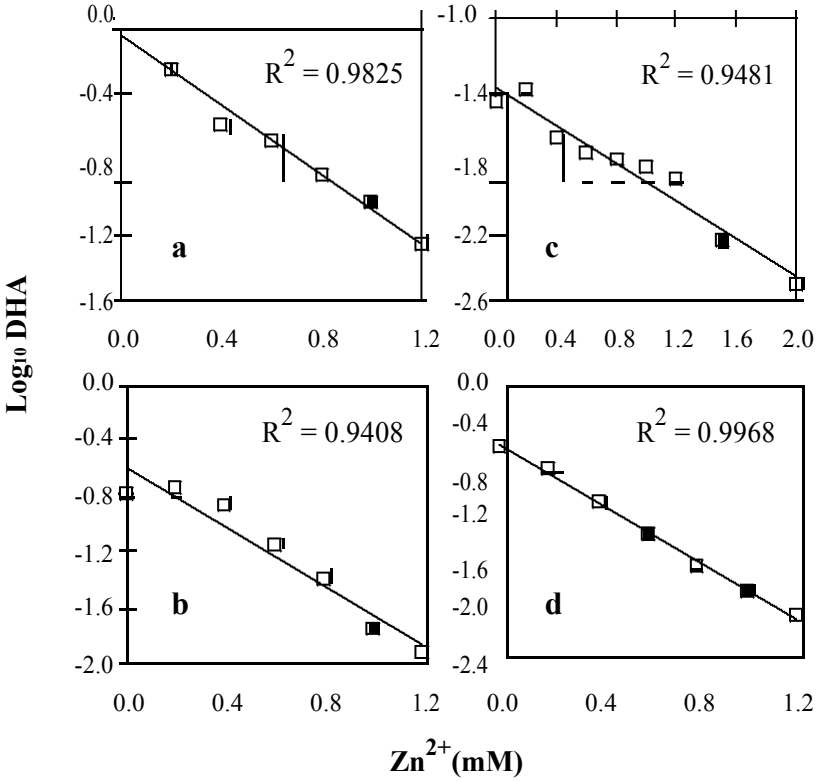

Figure 3. Correlation of Zn^{2+} concentrations with dehydrogenase activity (DHA) in response to zinc by *Escherichia* sp. PLK 1 (a), *Proteus* sp. PLK 2 (b), *Micrococcus* sp. PLK 4 (c) and *Pseudomonas* sp. PLK 5 (d). The data points represent mean (n=3) activity.

Micrococcus sp. PLK4. This variation may be due to differences in cell wall components or dehydrogenase systems, since different microorganisms have been reported to have different dehydrogenase systems (Praveen-Kumar, 2003). The rate of dehydrogenase activity is in the order: *Escherichia* sp. PLK1 > *Pseudomonas* sp. PLK5 > *Proteus* sp. PLK2 > *Micrococcus* sp. PLK4.

The effects of the different concentrations of Zn^{2+} on the bacterial isolates with respect to the dehydrogenase activity and its inhibition are shown in Figures 1 and 2. The responses of the bacterial dehydrogenase activities to Zn^{2+} is concentration-dependent and vary among the organisms. For *Escherichia* sp. PLK1 and *Pseudomonas* sp. PLK5, dehydrogenase activity reduced with increasing concentrations of Zn^{2+} (Figure 1). On the contrary, for *Proteus* sp. PLK2 and *Micrococcus* sp. PLK4, dehydrogenase activities were stimulated at 0.2 mM Zn^{2+} and thereafter progressive inhibition was also observed at concentrations above 0.2 mM. The tolerance of *Proteus* sp. PLK2 and *Micrococcus* sp. PLK4 observed at lower Zn^{2+} is attributable to the use of zinc as trace element by these bacteria. The inhibition of dehydrogenase activities observed in this study is consistent with the reported toxic effects of zinc at high concentrations (Ji and Silver, 1995). Although zinc is an essential trace element and plays an important role in the development, growth and differentiation of all living systems (Ohnesorge and Wilhelm, 1991), it is known to be a potent inhibitor of the respiratory electron transport system (Kasahara and Anraku, 1974; Beard et al., 1995). Results presented in Figure 2 showed that at lower Zn^{2+} concentrations (≤ 0.4 mM), *Escherichia* sp. PLK1 had higher percentage inhibition than other organisms. This implies that *Escherichia* sp. PLK1 was more sensitive to Zn^{2+} stress than the other bacterial strains studied. Comparatively, at higher concentrations (≥ 0.6 mM), *Micrococcus* sp. PLK4 was more tolerant to the Zn^{2+} than the other bacteria. Different sensitivities of microbes to zinc concentrations could be related to the genetic make up of the organism.

The dehydrogenase activity (DHA) correlated with Zn^{2+} concentration with R^2 values greater than 0.94 ($0.9408 \leq R^2 \leq 0.9968$) in all the bacterial strains (Figure 3). The relationships between the dehydrogenase activity and Zn^{2+} concentration are given as, Log_{10} DHA = -0.983 Zn^{2+} (mM) – 0.099, Log_{10} DHA = - 1.231 Zn^{2+} (mM) – 0.450, Log_{10} DHA = -0.538 Zn^{2+} (mM) – 1397 and Log_{10} DHA = - 1.236 Zn^{2+} (mM) – 0.575 for *Escherichia* sp. PLK1, *Proteus* sp. PLK2 *Micrococcus* sp. PLK4 and *Pseudomo-nas* sp. PLK5, respectively. The high R^2 values (> 0.94) observed with all the bacterial strains indicated that zinc

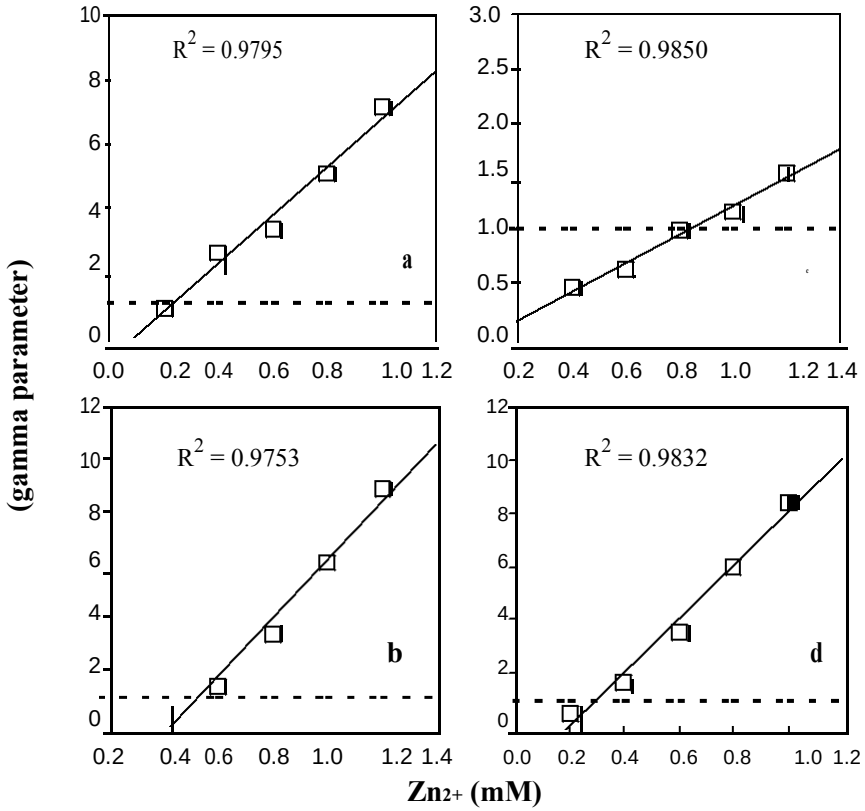

Figure 4. Linear regression of the gamma parameter ( ) values obtained from the mean inhibition data of zinc against *Escherichia* sp. PLK 1 (a), *Proteus* sp. PLK 2 (b), *Micrococcus* sp. PLK 4 (c) and *Pseudomonas* sp. PLK 5 (d). IC_{50} was calculated from the linear curves at = 1.

Table 2. Median and total inhibitory concentrations of zinc against the bacterial strains.

Bacteria	Inhibitory concentrations (mM)[a]	
	IC_{50}	IC_{100}
Escherichia sp. PLK1	0.236 ± 0.044	1.558 ± 0.037
Proteus sp. PLK2	0.529 ± 0.007	1.283 ± 0.068
Micrococcus sp. PLK4	0.864 ± 0.138	2.469 ± 0.045
Pseudomonas sp. PLK5	0.301 ± 0.024	1.328 ± 0.094

[a]Data represent mean ± standard deviation of triplicate estimates.

concentration was a strong determinant of the dehydrogenase activity. This indicated that increase in the concentration of Zn^{2+} will seriously affect carbon metabolism and respiratory activities in these bacterial strains.

The gamma parameter gave good linearization of the dose-response data with the R^2 values greater than 0.97 ($0.9753 \leq R^2 \leq 0.9850$) in all the bacterial strains (Figure 4). Similar plots (not shown) with ± 1 standard deviations of the values gave R^2 values greater than 0.95 ($0.9572 \leq R^2 \leq 0.9903$) in all the bacterial strains. Table 2 shows the IC_{50} of Zn^{2+} estimated from the gamma parameter plot at = 1 and the IC_{100} of Zn^{2+} estimated from the logarithmic transformation of the inhibition data (plots not shown) for the various isolates. *Escherichia* sp. PLK1 having the least IC_{50} of 0.236 mM was the most sensitive to Zn^{2+} while *Micrococcus* sp. PLK4 having the highest IC_{50} of 0.864 was most tolerant. A 50% effective concentration (EC50) of zinc on the microcosm sediment communities has been reported as 0.92 and 1.53 mM using acetate incorporation and glucosidase activity assays, respectively (Barnhart and Vestal, 1983). A much lower EC50 (0.05 mM) was reported for a natural microbial community in compost using acetate incorporation (Barnhart and Vestal, 1983). The differences could be attributed to the method of analysis, zinc salt used, growth medium or microbial species.

Tolerance of zinc (as $ZnSO_4$) have been studied in two zinc-solubilizing bacteria, *Bacillus* sp. ZSB-O-1 and *Pseudomonas* sp. ZSB-S-2. The tolerance limits for these bacteria was determined to be up to 100 mg/l (≈ 1.53 mM) of Zn^{2+} in an *in vitro* broth asssay (Vankatakrishnan et al., 2003). Total inhibition of nitrification process at 1.2

mg/l (≈ 0.02 mM) was reported for an autotrophic biomass in an activated sludge (Juliastuti et al., 2003). Higher zinc tolerance have been reported in *Escherichia coli* and *Pseudomonas aeruginosa*. A 10 mM concentration did not significantly affect the survival of *P. aeruginosa* but decreased the survival of *E.coli* (Babich and Stotzky, 1978). Minimum inhibitory concentrations (MICs) as large as 24 and 48 mM was previously reported for *P. aeruginosa* isolated from natural waters (de Vincente et al., 1990). A 5 h minimum bactericidal concentration of zinc greater than 64 mM have been reported for planktonic and biofilm *P. aeruginosa* (Teitzel and Parsek, 2003). MIC of 1 mM was reported by Mergeay et al. (1985) for *E. coli*. The high tolerance of zinc observed in these reports could be attributed to zinc resistance in the bacterial strains. The bacterial strains of the New Calabar River seem to have normal sensitivity with variable inhibition at low concentrations and almost total inhibition at concentrations ranging from 1.2 to 2.0 mM Zn^{2+}. The 2-way ANOVA showed that the dehydrogenase activity varied significantly ($p < 0.01$) with bacteria type and the concentrations of zinc.

The result of the *in vitro* study indicated that Zn^{2+} is potentially toxic to the bacterial strains of New Calaber River. Contamination and accumulation of Zn^{2+} in the river would therefore pose serious threat to the metabolic activities of the individual bacterial strain. However, the result does not indicate the response of the complex microbial community of the river to toxicity of zinc.

REFERENCES

Aoyama M, Nagumo T (1997). Effects of heavy metal accumulation in apple orchard soils on microbial biomass and activities. Soil Sci. Plant Nutr. 43: 821-831.

Babich H, Stotzky G (1978). Toxicity of zinc to fungi, bacteria and coliphages: influence of chloride ions. Appl. Environ. Microbiol. 36: 906-914.

Barnhart CLH, Vestal JR (1983). Effects of environmental toxicants on metabolic activity of natural microbial communities. Appl. Environ. Microbiol. 46: 970-977.

Beard SJ, Hughes MN, Poole RK (1995). Inhibition of the cytochrome bd-terminated NADH oxidase system in *Escherichia coli* K-12 by divalent metal cations. FEMS Microbiol. Lett. 131: 205-210.

Bidwell JP, Spotte S (1985). Artificial Seawaters: Formular and Methods. Jones and Barlett Publishers, Inc. Boston.

de Vincente A, Avilès M, Codina JC, Borrego JJ, Romero P (1990). Resistance to antibiotics and heavy metals of *Pseudomonas aeruginosa* isolated from natural waters. J. Appl. Bacteriol. 68: 625-632.

Goldman CR, Horne AJ (1983). Limnology. McGraw-Hill, New York.

Gong P (1997). Dehydrogenase activity in soil: a comparison between the TTC and INT assay under their optimum conditions. Soil Biol. Biochem. 29: 211-214.

Griffiths RP (1983). The importance of measuring microbial enzymatic functions while assessing and predicting lond-term anthropogenic perturbations. Mar. Pollut. Bull. 14: 162-165.

Hanson PJ, Evans DW, Colby DR,Zdanowicz VS (1993). Assessment of elemental contamination in estuarine and coastal environments based on geochemical and statistical modelling of sediments. Mar. Environ. Res. 36: 237-266.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994). Bergey's Manual of Determinative Bacteriology, 9th ed. Williams, Wilkins. Baltimore.

Horsfall JrM, Spiff AS (2002). Distribution and partitioning of trace metals in sediments of the lower reaches of the New Calabar River, Port Harcourt, Nigeria. Environ. Monitor. Assess. 78: 309-326.

Ji G, Silver S (1995). Bacterial resistance mechanism for heavy metals of environmental concern. J. Ind. Microbiol. 14: 64-168.

Juliastuti SR, Baeyens J, Creemers C, Bixio B, Lodewyckx E (2003). The inhibitory effects of heavy metals and organic compounds on the net maximum specific growth rate of the autotrophic biomass in activated sludge. J. Hazard. Mater. 100: 271-283.

Kasahara M, Anraku Y (1974). Succinate and NADH oxidase systems of *Escherichia coli* membrane vesicles: mechanism of selective inhibition of the system by zinc ions. J. Biochem. 76: 967-976.

Kelly JJ, Tate RL (1998). Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. J. Environ. Qual. 27: 609-617.

Kim C-W, Koopman B, Bitton G (1994). INT-Dehydrogenase activity test for assessing chloride and hydrogen peroxide inhibition of filamentous pure cultures and activated sludge. Water Res. 28: 1117-1121.

Klimkowicz-Pawlas A, Maliszewska-Kordybach B (2003). Effect of anthracene and pyrene on dehydrogenases activity in soils exposed and unexposed to PAHs. Water, Air and Soil Pollut. 145: 169-186.

Leppard GG (1981). Trace Element Speciation in Surface Waters. Plenum Press, New York.

Maliszewska-Kordybach B, Smreczak B (2003). Habitat function of agricultural soils as affected by heavy metals and polycyclic aromatic hydrocarbons contamination. Environ. Int. 28: 719- 728.

Mathew M,Obbard JP (2001). Optimization of the dehydrogenase assay for measurement of indigenous microbial activity in beach sediments contaminated with petroleum. Biotechnol. Lett. 23: 227- 230.

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, van Gijsegem F (1985). *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-borne resistance to heavy metals. J. Bacteriol. 162: 328-334.

Nürnberg HW (1984). The voltammetric approach in trace metal chemistry of natural waters and atmospheric precipitation. Anal. Chem. Acta 164: 1-21.

Odokuma LO, Abah AE (2003). Heavy metal biosorption by three bacteria isolated from a tropical river. Global J. Environ. Sci. 2: 98-101.

Odokuma LO, Ijeomah SO (2003a). Tolerance of bacteria to toxicity of heavy metals in the New Calabar River. Glob. J. Environ. Sci. 2(2): 128-132.

Odokuma LO, Ijeomah SO (2003b). Seasonal changes in the heavy metal resistant bacterial population of the New Calabar River. Global J. Pure and Appl. Sci. 9: 425-433.

Odum EP (1985). Trends expected in stressed ecosystems. Bioscience 35: 419-422.

Ohnesorge FK, Wilhelm M (1991). Metals and their Compounds in the Environment (Mariam E. ed.), VGH, Germany.

Praveen-Kumar, JCT (2003). 2,3,5-Triphenyltetrazolium chloride (TTC) as electron acceptor of culturable soil bacteria, fungi and actinomycetes. Biol. Fertil. Soils 38: 186 – 189.

Purves D (1985). Trace-Elements Contamination of the Environment. Elsevier, Armsterdam.

Teitzel, GM, Parsek MR (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 69: 2313-2320.

Vankatakrishnan SS, Sudlayandy RS, Savariappan AR (2003). Assessing in vitro solubilization potential of different zinc solubilizing bacteria (ZSB) isolates. Brazilian J. Microbiol. 34: 121-125.