TOXICITY OF ZINC TO HETEROTROPHIC BACTERIA FROM A TROPICAL RIVER SEDIMENT

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Abstract. Tolerance to Zn^{2+} by pure cultures of *Bacillus*, *Salmonella* and *Arthrobacter* species isolated from New Calabar River sediment was assessed through dehydrogenase assay. The cultures were exposed to Zn^{2+} concentrations of 0.2 to 2.0 mM in a nutrient broth-glucose-TTC medium. The responses of the bacterial strains varied with Zn^{2+} concentration. In *Salmonella* sp. SED2, Zn^{2+} stimulated dehydrogenase activity at 0.2 mM. In *Bacillus* sp. SED1 and *Arthrobacter* sp. SED4, dehydrogenase activity was progressively inhibited with increasing Zn^{2+} concentration. The IC_{50} ranges from 0.206 ± 0.030 to 0.807 ± 0.066 mM. Total inhibition of dehydrogenase activity was observed at concentrations ranging from 1.199 ± 0.042 to 1.442 ± 0.062. The order of zinc tolerance is: *Salmonella* sp. SED2 > *Arthrobacter* sp. SED4 > *Bacillus* sp. SED1. The result of the in vitro study indicated that Zinc is potentially toxic to sediment bacteria and could pose serious threat to their metabolism in natural environments.
Keywords: *dehydrogenase activity, sediment bacteria, New Calabar River.*

Introduction

Bacteria and other microorganisms densely colonize freshwater and marine sediments. In these environments, bacteria constitute the primary agents of early transformation of organic matter and regeneration of nutrients and also serve as food source for higher trophic level.

Microorganisms are vital for the efficient functioning of any ecosystem, hence factors that affect their metabolism, composition and abundance are of great concern. Monitoring microbial responses has been recommended as an early warning indicator of ecosystem stress as microbes respond promptly to environmental perturbations [12, 29]. Measurement of microbial enzyme activity is used in the assessment of ecotoxicological impacts of environmental substrates. In this regard, dehydrogenase activity has been widely used. The dehydrogenase assay is an effective primary test for assessing the potential toxicity of metals to soil microbial activities [1, 7, 18, 31] and bioavailability of metal in a beach sediment [9].

The pollution of New Calabar River is due to anthropogenic activities along its bank [25, 27, 28]. The heavy metal content, seasonal variations in the population of heavy metal resistant bacteria and toxicity of heavy metals to bacteria isolated from the New Calabar River have been reported [23, 25, 26]. Moreso, Odokuma and Abah reported bioaccumulation of selected heavy metals by bacteria isolated from this river [24]. Although, these works focused on pure cultures of bacterial isolates, they did not consider the inhibition of dehydrogenase enzyme activity in these bacteria. This study was aimed at assessing the effects of zinc on the dehydrogenase activities of *Bacillus*, *Salmonella* and *Arthrobacter* species isolated from New Calabar River sediment.

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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 is situated in the Niger delta of Nigeria. The water is brackish and impacted by effluent discharges from industries sited along its bank. The sampling site have been described elsewhere [26, 27]. Sediment and water samples were collected along the course of the river at Choba. Eckman grab sampler was used for collection of sediment sample. The overlying water sample was collected midstream along the course of the river from a depth of 30 cm. The samples were collected in sterile glass bottles, stored in a cooler and taken to laboratory. All samples were analysed within 6 hours of collection. The pH and zinc content of the samples 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 New Calabar River sediment were isolated and purified on nutrient agar plates. Characterizations were done using standard microbiological methods. Identifications to the generic level followed the schemes of Holt *et al*. [13].

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 ^{o}C). The cells were harvested by centrifugation at 4000 rpm for 10 minutes. Harvested cells were washed twice in deionized distilled water and resuspended in the same water. The resuspended cells were standardized in a spectrophotometer to an optical density of 0.85 at 420nm. The dry weights of the standardized cells were determined by drying 10 ml of cell suspension to constant weight in an oven at 110^{o}C. These standardized cell suspensions were used as inoculum in the dehydrogenase activity assay.

Dehydrogenase activity assay

Dehydrogenase activity was determined using TTC as the artificial electron acceptor, which is reduced to the red-coloured TPF. The assay was done in 3-ml volumes of nutrient broth-glucose-TTC medium supplemented with varying concentrations (0.2 – 1.0 mM) of Zn^{2+} as zinc sulphate in separate 20 ml 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 ^{o}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 and 1.0 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 statically at room temperature (28 ± 2 ^{o}C) for 4 h. The TPF produced were 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 [0-50 mg/l TPF (Sigma) in amyl alcohol; R^2 = 0.996]. Dehydrogenase activity was expressed as milligrams of TPF formed per mg dry weight of cell biomass per hour.

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Zinc inhibition of dehydrogenase activity was calculated relative to the control. The percentage inhibitions for *Bacillus* and *Arthrobacter* species were linearized against the concentrations of Zn^{2+} using gamma parameter (Γ) as shown in the equation below [19]. The toxicity threshold concentrations (IC_{20} and IC_{50}) were then determined from regression plots. The total inhibitory concentrations (IC_{100}) were estimated from the linear regressions of log transformation plots of the dose-response data.

Inhibition of dehydrogenase activities in the sediment bacteria was compared with that of planktonic bacteria isolated from New Calabar River water.

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

Statistical analysis

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

Results and discussion

The sediment is slightly acidic (pH 6.62) and has elevated zinc concentrations of 65.8 mg/kg sediment. The pH and zinc content of the overlying river water were 6.4 and 5mg/l (≈76.48 μM) respectively. The higher zinc content in the sediment could be attributed to incorporation of zinc into sediment following it's association with particulate matters. In an undisturbed environment, heavy metals are preferentially transferred from the dissolved phase and thus metal concentrations in sediments are generally much higher than in the overlying water [6]. Previously, zinc levels of 0.01 to 0.71 mg/l [24, 25, 26] in the river water and 31.18 to 32.02 mg/kg [15] in the sediment of New Calabar River was reported. This indicated that zinc was accumulating in the New Calabar River water and sediment over time.

Three predominant bacterial strains comprising one Gram negative (*Salmonella* sp. SED2) and two Gram positive (*Bacillus* sp. SED1 and *Arthrobacter* sp. SED4) organisms were isolated from the river sediment. These isolates were able to reduce TTC to its formazan and so were used to assess toxicity of toxicant through dehydrogenase activity assay. *Bacillus*, *Arthrobacter*, *Salmonella* and *Proteus* species have been isolated from New Calabar River by Odokuma and Ijeomah [24, 25].

The rate of dehydrogenase activity varied among the bacterial strains (*Table 1*). The Gram positive *Bacillus* sp. SED1 had higher rates of dehydrogenase activity than the Gram negative *Salmonella* sp. SED2 and Gram positive *Arthrobacter* sp. SED4. In a similar study with planktonic bacteria of New Calabar River, Gram negative bacteria was reported to have higher rate of dehydrogenase activity than the Gram positive ones [23]. The reason for these differences is not known. However, it may be attributed to the physiology of the bacteria.

The effects of Zn^{2+} on the dehydrogenase activity and its relative inhibition in the bacterial strains are shown in *Figure 1* and *Table 2* respectively. In *Bacillus* sp. SED1 and *Arthrobacter* sp. SED4, dehydrogenase activity decreased with increasing concentration of Zn^{2+}. In *Salmonella* sp. SED2, dehydrogenase activity was slightly stimulated at 0.02 mM Zn^{2+} and progressively inhibited at concentrations greater than 0.2 mM (0.4 – 1.0 mM). The stimulatory effect observed with *Salmonella* sp. SED2

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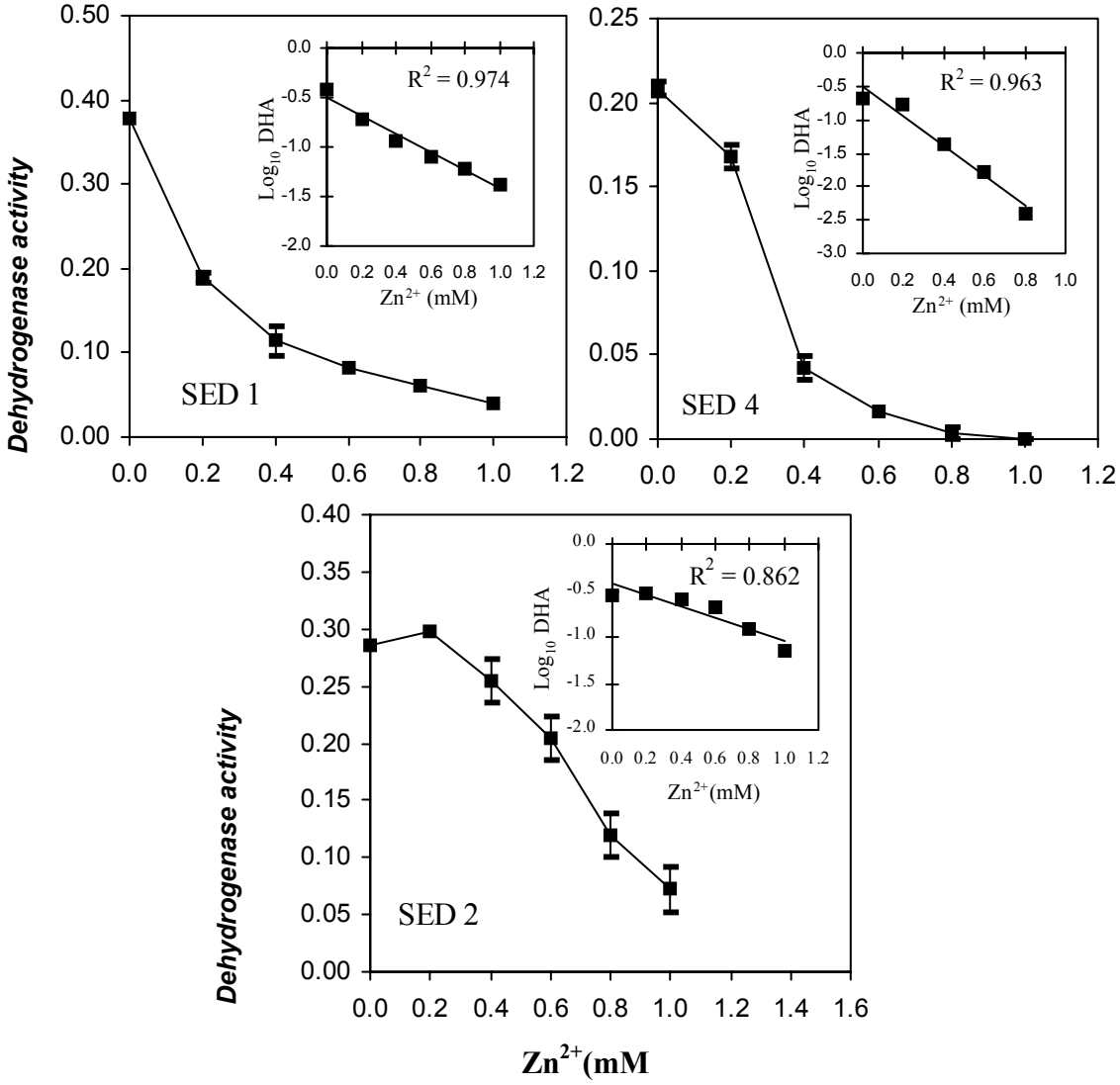

Figure 1. *Dehydrogenase activity in response to various concentrations of zinc ion by Bacillus sp. SED1, Salmonella sp. SED2 and Arthrobacter sp. SED4. The vertical bars indicates mean ± standard deviation (n=3). The figure insets show linear relationships between Zn^{2+} and mean dehydrogenase activity (DHA) in each bacterial strain*

Table 1. *Uninhibited dehydrogenase activities in the isolates*

Strain	Dehydrogenase activity[a] (mg Formazan/mg cell dry wt/h)
Bacillus sp. SED1	0.379 ± 0.038
Salmonella sp. SED2	0.208 ± 0.004
Arthrobacter sp. SED4	0.285 ± 0.002

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may be attributed to the use of zinc as trace element by this bacterium. Zinc is associated with a number of processes essential for growth and metabolism in bacteria [8]. The inhibition of dehydrogenase activity observed in this study is consistent with the reported toxic effect of zinc at high concentrations [16]. Although zinc is an essential element, it is an inhibitor of respiratory activities in microorganisms [3,17, 30].

Results presented in *Table 2* showed that the Gram negative *Salmonella* sp. SED2 tolerated zinc more than the Gram positive *Bacillus* sp. SED1 and *Arthrobacter* sp. SED4. *Salmonella* sp. SED2 had lower percentage inhibition of dehydrogenase activity at all concentrations of Zn^{2+}. Better tolerance to heavy metal toxicity by Gram negative bacteria have been reported [20,21]. In comparison, there is no significant difference ($p < 0.05$) between the percentages of inhibition of dehydrogenase activities in sediment and planktonic bacteria of New Calabar River. This did not corroborate reports that organisms isolated from heavy metal polluted habitats are more tolerant to metals than organisms isolated from unpolluted habitats. It could be that the concentration of zinc was not high enough to select for tolerant organisms in the sediment. The effects of zinc on the microbial activity of water and sediment communities have been reported [22, 23, 32]. A suppression of organic decomposition was observed in the heavy metal contaminated sediment of Palestine Lake containing average zinc level of 17840 mg/kg sediment [22]. Likewise, Zn^{2+} inhibited glucose uptake and mineralization by water and sediment microbial communities of a contaminated stream. A 10 % reduction in the maximum rate of glucose uptake was obtained at lower metal concentrations in the water samples than in the sediment ones [32]. This indicates that water microbial community is more sensitive to metal toxicity than sediment community. Hornor and Hilt have made similar observations, where bacteria in polluted site are more tolerant to metal than those in unpolluted sediment [14]. Hornor and Hilt also observed that the presence of Zn-tolerant bacteria correlated with the degree of heavy metal contamination. Numerous other reports also revealed that bacteria isolated from environments with high levels of heavy metal exhibit greater metal tolerance than bacteria isolated from unpolluted habitats [2, 10, 11, 33].

The dehydrogenase activities correlated with Zn^{2+} concentration as shown in *Figure 1* (insets). The high R^2 values ($0.862 \leq R^2 \leq 0.974$) indicated that Zn^{2+} concentration was a strong determinant of dehydrogenase activities in the organisms. Thus , the organisms are at serious stress at high concentrations of Zn^{2+}.

The gamma parameter model gave good linearization of the dose response data for *Bacillus* sp. SED1 ($0.987 \leq R^2 \leq 0.994$) and *Arthrobacter* sp. SED4 ($0.976 \leq R^2 \leq 0.993$). However, for *Salmonella* sp. SED2, the response (percentage inhibition) is linear ($0.970 \leq R^2 \leq 0.993$) with the concentrations of Zn^{2+} and does not require linearization process (*Figure 2*). *Table 3* shows the toxicity threshold concentrations (IC_{20}, IC_{50} and IC_{100}) of Zn^{2+} estimated from the linear regression models. *Bacillus* sp. SED1 having the least IC_{20} and IC_{50} of 0.071 and 0.206 mM respectively was the most sensitive to Zn^{2+} while *Salmonella* sp. SED1 having the highest IC_{20} and IC_{50} of 0.488 and 0.807 mM respectively was the most tolerant. Using INT-dehydrogenase activity assay, Pérez-Garcia and co-workers reported an IC_{20} and IC_{50} of 0.999 and 2.88 mM of Zn^{2+} against *Pseudomonas fluorescens* [30]. In a MetPLATE ™ assay system (based on β-galactosidase activity), zinc IC_{50} of 0.11 ± 0.001 mg/l (0.002mM) was reported for *Escherichia coli* [5]. In a growth inhibition test assessed via turbidity measurements, 7.15 mg/l Zn^{2+} (0.12mM) inhibited the growth of *Pseudomonas putida* by 50 % [34]. In

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Table 2: *Zinc inhibition of dehydrogenase activity in sediment and planktonic bacterial species*

Bacterial strains	Inhibition (%)[a]				
	Zn^{2+} (mM)				
	0.2	0.4	0.6	0.8	1.0
Sediment bacteria					
Bacillus sp. SED1	47.804 ± 1.650	66.883 ± 4.496	75.162 ± 3.240	80.562 ± 1.052	84.881 ± 1.080
Salmonella sp. SED2	-4.000 ± 0.000	10.000 ± 6.000	26.000 ± 6.000	53.333 ± 6.110	68.667 ± 6.429
Arthrobacter sp. SED4	17.981 ± 3.155	74.763 ± 3.155	86.330 ± 1.821	91.588 ± 0.911	93.691 ± 0.000
Planktonic bacteria					
Escherichia sp. PLK 1	44.523 ± 2.845	71.313 ± 4.167	76.292 ± 3.580	83.404 ± 0.822	87.672 ± 0.410
Proteus sp. PLK 2	–7.453 ± 3.000	21.140 ± 3.037	57.588 ± 3.037	76.806 ± 3.037	85.421 ± 3.037
Micrococcus sp. PLK 4	–18.182 ± 3.445	31.034 ± 3.449	37.931 ± 3.448	49.425 ± 1.991	53.448 ± 4.562
Pseudomonas sp. PLK 5	33.244 ± 2.053	62.376 ± 5.393	77.599 ± 4.107	85.573 ± 1.480	89.247 ± 1.344

[a] *Data represent means ± standard deviation of triplicate tests*

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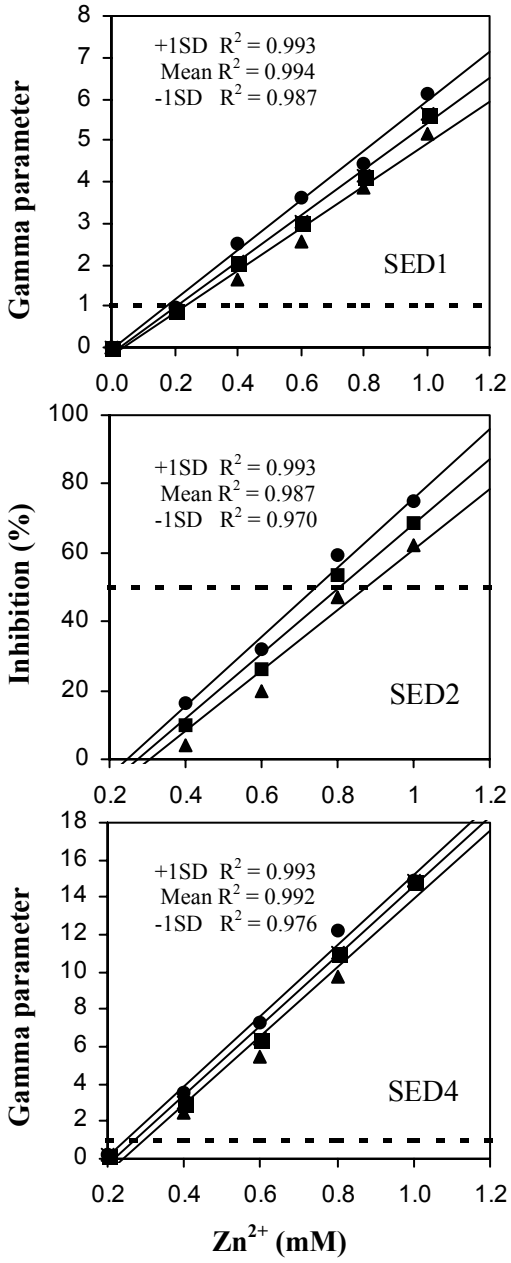

Figure 2. *Relationships between the mean ± standard deviation of gamma parameter and percent inhibition values with zinc ion concentrations for Bacillus sp. SED1, Salmonella sp. SED2 and Arthrobacter sp. SED 4. IC_{50} was calculated as mean ± standard deviation from the linear curves. Symbols: Circle = +1SD, Square = mean, Triangle = -1SD.*

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a similar growth inhibition test, 0.1 and 0.5mM of zinc inhibited growth of *Streptococcus faecalis* by 8.77 and 18.42 % respectively [4].

The 2-way ANOVA show that the dehydrogenase activity and its percentage inhibition varied significantly ($p < 0.05$) with bacteria type and the concentrations of zinc.

The result of the *in vitro* study indicated that Zn^{2+} is potentially toxic to the sediment bacteria of New Calaber River. Contamination and accumulation of Zn^{2+} in the sediment would likely impact negatively on carbon metabolism and respiratory activities of the bacterial strains. Since bacteria play important role in detrital breakdown and nutrient cycling, disturbances in their activity would result in general ecosystem stress.

Table 2. *Threshold inhibitory concentrations of zinc against sediment bacterial strains*

Bacteria	Inhibitory concentrations (mM)[a]		
	IC_{20}	IC_{50}	IC_{100}
Bacillus sp. SED1	0.071 ± 0.019	0.206 ± 0.030	1.442 ± 0.062
Samonella sp. SED2	0.488 ± 0.045	0.807 ± 0.066	ND
Arthrobacter sp. SED4	0.233 ± 0.023	0.273 ± 0.024	1.199 ± 0.042

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