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Kinetics of dose-response relationship of heavy metals with dehydrogenase activity in wastewater bacteria

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ABSTRACT:

Toxicity of Zn^{2+}, Cd^{2+} and Co^{2+} to *Escherichia coli, Pseudomonas* and *Bacillus* species isolated from petroleum refinery effluent was assessed using dehydrogenase activity (DHA) inhibition test. Exposure of the cells to the metal ions resulted in inhibition of dehydrogenase activity. The median inhibitory concentration of the metal ions ranged from 0.0554 to 0.3883 mM (Zn^{2+}), 0.0279 to 0.3004 mM (Cd^{2+}) and 0.0013 to 0.2778 mM (Co^{2+}). The trends of the inhibitory effects could be mathematically described with logistic and sigmoid dose-response models and in a manner similar to the non-competitive inhibition of enzymes. The threshold concentration above which toxic effect is observed ranged from 0.0013 mM (Zn^{2+} against *Pseudomonas sp.* RWW2) to 0.05 mM (Zn^{2+} against *Escherichia coli*). In terms of non-competitive inhibition of dehydrogenase activity, the threshold concentration ranged from 0.0183 mM (Cd^{2+} against *Pseudomonas sp.* DAF1) to 0.05 mM (Zn^{2+} against *Escherichia coli*). The coefficients of inhibition *Ki* correlated with the IC_{50}, thus they are suitable parameters for kinetic analyses of metal toxicity against bacteria.

Keywords:
Dehydrogenase activity, heavy metals, dose-response models, toxicity.

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INTRODUCTION

Heavy metals are widely distributed and persistent environmental pollutants that are introduced into the environment through industrial activities. Heavy metals contaminate natural habitats and alter macro and microbiological communities (Purves, 1985; Davies *et al.,* 1991; Binning and Baird, 2001; Horsfall and Spiff, 2002; Nweke *et al*., 2006; 2007a). Some heavy metals (e.g Cd and Hg) have no known physiological function and are toxic even at low concentrations. Others (e.g Cu, Ni, Zn and Co) are essential trace elements required for normal physiological function of microorganisms. The toxic effects of metals include; blocking of functional groups, displacement or substitution of essential metal ions from biomolecules, conformational modifications, denaturation and inactivation of enzymes and disruption of membrane integrity (Gadd, 1993; Doelman *et al.,* 1994; Li and Tan 1994).

Microorganisms are vital for efficient functioning of any ecosystem, thus factors affecting their metabolism and diversity are of great concern. Microbes respond promptly to environmental pollution and monitoring microbial responses have been recommended as an early warning indicator of ecosystem stress (Griffiths, 1983; Odum, 1985). Microbial parameters including growth rate (Juliastuti *et al*., 2003b), biomass measurement (Guckert, 1996), inhibition of bioluminescence (Ren and Frymier, 2003) and activity of specific and non-specific enzymes (Bitton *et al*., 1992) may be used to evaluate toxic effects of metals on bacterial populations.

Enzymes are key catalysts of metabolic reactions in cells and their inhibition by metals has been explored as basis for ecotoxicity testing. A wide range of enzymes has been considered with special emphasis on dehydrogenases (Bitton and Koopman 1986; Obst *et* al., 1988; Montuelle *et al*., 1994; Codina *et al*., 1994; Nweke *et al*., 2006, 2007a,b; Orji *et al*., 2008). Dehydrogenase activity assay is also an effective primary test for assessing toxicity of phenols (Nweke and Okpokwasili, 2010a, b; Nwanyanwu and Abu, 2011) and plant extracts (Alisi *et al*., 2011) against bacteria. However, the kinetics of toxicity has not been widely studied. The objective of this study was to compare the effects of zinc, cadmium and cobalt on the dehydrogenase activity in petroleum refinery effluent bacteria, using kinetic analysis.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains were isolated from wastewater and wastewater treatment system of Port Harcourt petroleum refinery, Port Harcourt, southeastern Nigeria**.** The method of isolation and identification of the bacterial strains was as described elsewhere (Nweke and Okpokwasili, 2010a).

Dehydrogenase assay

Dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride as the artificial electron acceptor, which is reduced to 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+}, Cd^{2+} and Co^{2+} as $ZnSO_4$, $CdCl_2$ and $CoCl_2$ respectively in separate screw-capped test tubes. Portions (0.3 ml) of washed bacterial suspensions (A_{420} = 0.5) were inoculated into triplicate glass tubes containing 2.5 ml of phthalate-buffered (pH 6) nutrient broth glucose medium amended with Zn^{2+}, Cd^{2+} and Co^{2+} and 0.2 ml of 0.4% (w/v) TTC in deionized distilled water was added to each tube to obtain final concentrations of 0.0.05 - 1.4 mM. The final concentrations of nutrient broth and glucose in the medium were 2 mg/ml each. The controls consisted of the isolates and the media without metal. The reaction mixtures were incubated under static conditions at room temperature (28 ± 2°C) for 24 h. The TPF produced was extracted in 4 ml of amyl alcohol and determined

spectrophotometrically. The dehydrogenase activities as µg TPF/mg of dry cell wt/h and percentage inhibitions were computed.

Kinetic modelling

The enzyme activity (EA) and inhibition of the enzyme activity (INH) relative to controls are given by the expressions:

where

$$\text{EA}(\%\text{of control}) = \frac{T_A}{C_A} \times 100 \quad (1)$$

$$\text{INH}\ (\%) = \frac{C_A - T_A}{C_A} \times 100 \quad (2)$$

C_A: is absorbance of TPF produced in control test (without metal)

T_A: is absorbance of TPF produced in the test with different concentrations of metal

The enzyme activity (% of control) can be described by the logistic function:

$$\text{EA}(\%\text{of control}) = \frac{a_m}{1 + \left(\frac{I}{b}\right)^c} \quad (3)$$

Where

a_m: is maximum enzyme activity (% of control)

b: is slope parameter indicating the inhibition rate, IC_{50} (mM)

c: is a dimensionless inhibition parameter

I: is inhibitor concentration (mM)

But:

$$\text{INH}(\%) = 100 - \text{EA}\ (\%\text{of control}) \quad (4)$$

Substituting equation-3 into equation-4 yields:

$$\text{INH}\ (\%) = 100 - \frac{a_m}{1 + \left(\frac{I}{b}\right)^c} \quad (5)$$

Assuming there is no stimulation of enzyme activity at low concentrations of metal ion, a_m becomes 100 %, thus equation-5 becomes:

$$\text{INH}(\%) = 100 - \frac{100}{1 + \left(\frac{I}{b}\right)^c} \quad (6)$$

Rearranging equation-6 yields:

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \left(\frac{I}{b}\right)^c}\right) \times 100 \quad (7)$$

Equation-7 can be rewritten as:

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \frac{I^c}{b^c}}\right) \times 100 \quad (8)$$

Equation 8 is equivalent to equation 9 reported in literature (Kroiss *et al*., 1992; Juliastuti *et al*., 2003a). Where, $c = KI$ and $b^c = K_i$. K_i is the coefficient of inhibition (mM).

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \frac{I^{KI}}{K_i}}\right) \times 100 \quad (9)$$

Incorporating the threshold concentration *a* (concentration of metal above which toxic effect is observed) in equation 8 and 9 yields equation 10 and 11 respectively. Equations 10 and 11 are similar to equation 12 originally proposed by Ren and Frymier (2003) to describe inhibition of bioluminescence by metals.

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \frac{(I-a)^c}{b^c}}\right) \times 100 \quad (10)$$

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \frac{(I-a)^{KI}}{K_i}}\right) \times 100 \quad (11)$$

$$\text{INH}(\%) = \left(1 - \frac{1}{1 + \frac{I}{K_i} - \frac{a}{K_i}}\right) \times 100 \quad (12)$$

Equation 12 assumes that metal ions repress bacterial dehydrogenase activity by inhibiting the rate-determining step in the formation of triphenyl formazan in a manner similar to non-competitive inhibition of enzymes. Inhibitions of enzyme activity data derived from equation 2 are fitted into equations 9, 11 and 12. The kinetic parameters were estimated by iterative minimization of least squares using Levenberg-Marquardt algorithm of Table Curve 2D. Regression was done using the mean data and standard deviations. The toxicity thresholds, IC_{20}, IC_{50} and IC_{80} which are the concentrations of metals that inhibited dehydrogenase activity by 20, 50 and 80% respectively were estimated from equation 9. The Pearson product-moment correlation and analysis of variance (ANOVA) were done using Microsoft Excel 2003.

RESULTS AND DISCUSSION

The effect of zinc, cadmium and cobalt on the production of triphenyl formazan in test bacteria is shown in Figure-1. The relative effects of these metals in terms of percentage inhibition of dehydrogenase activity in the bacterial isolates are shown in Figures 2 - 5. In all the bacterial strains, cobalt, zinc and cadmium inhibited dehydrogenase activity. The inhibitions increased with increase in the concentration of metal. The inhibitions were relatively less pronounced at lower concentrations of zinc than with other metals. However, at 0.05 mM, zinc inhibited dehydrogenase activity in *Bacillus sp.* DISK1 by 43.666 ± 1.523%. Generally, cadmium and cobalt are more toxic to bacterial dehydrogenases at 0.05 mM than zinc. However, it is noteworthy that cadmium stimulated dehydrogenase activity in *Pseudomonas sp.* RWW2 by 6.613 ± 4.675% at 0.05 mM. Cobalt inhibited dehydrogenase activity in *Pseudomonas sp.* DAF1, *Pseudomonas sp.* RWW2, *Bacillus sp.* DISK1 and *Escherichia coli* by 45.669 ± 1.181, 17.679 ± 2.337, 43.666 ± 1.523 and 66.410 ± 8.442 respectively at 0.05 mM. Cadmium exhibited similar levels of inhibition in the bacterial

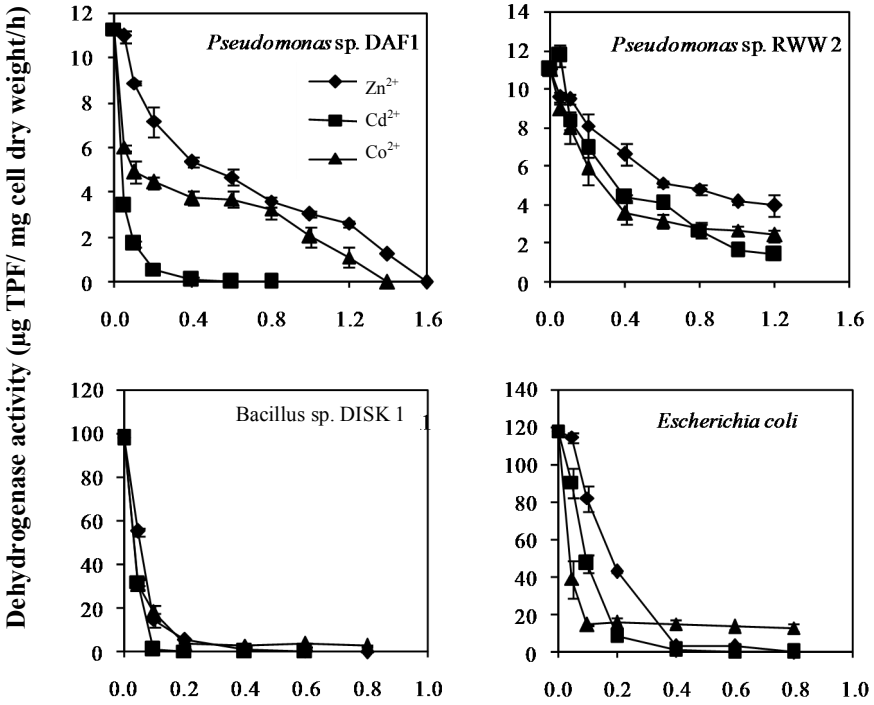

Figure 1: Production of triphenyl formazan in response to metal toxicity in pure cultures of wastewater bacteria

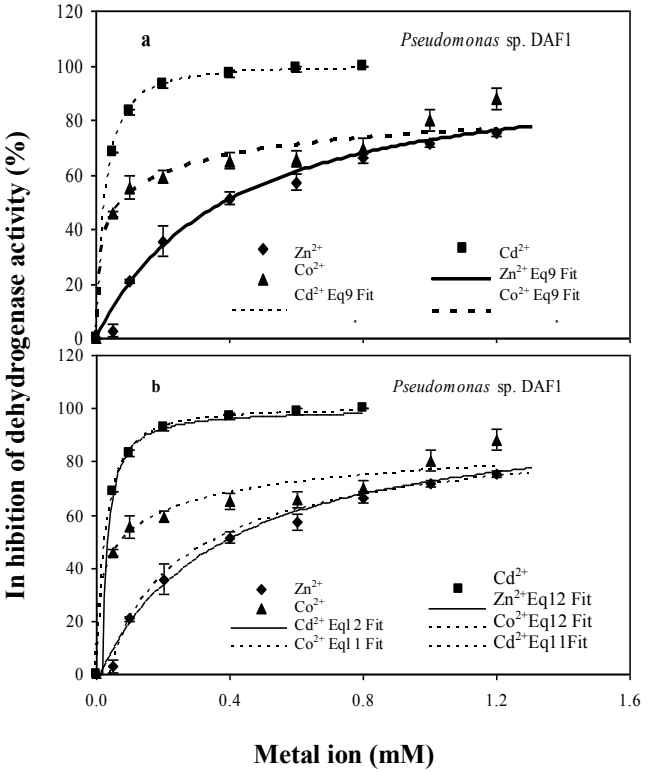

Figure 2: Effects of metals on dehydrogenase activity in *Pseudomonas sp.* DAF1. Experimental data mean ± 1SD (n = 3) as data points and bars are shown with predicted values derived from equation 9 (a) and equations 11 and 12 (b).

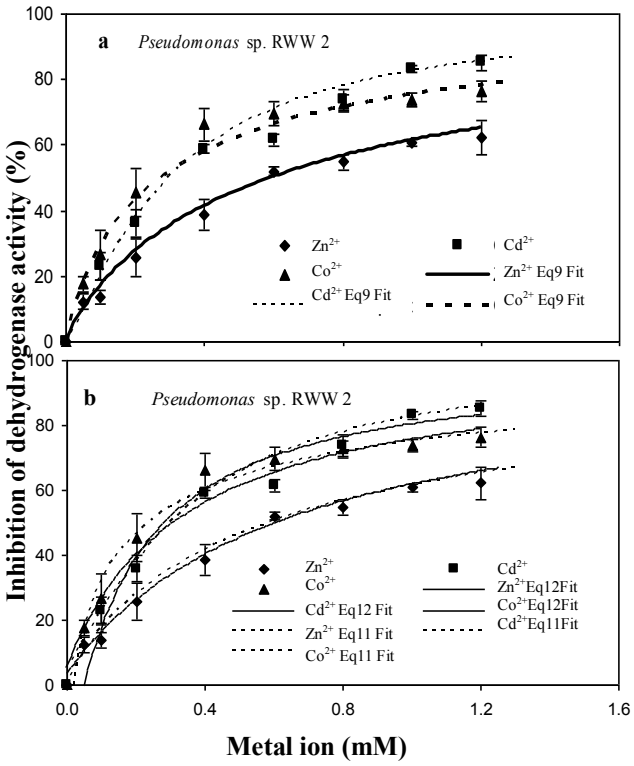

Figure 3: Effects of metals on dehydrogenase activity in *Pseudomonas sp.* RWW2. Experimental data mean ± 1SD (n = 3) as data points and bars are shown with predicted values derived from equation 9 (a) and equations 11 and 12 (b)

strain at 0.05 mM with *Bacillus sp.* DISK1, *Pseudomonas sp.* DAF1 and *Escherichia coli* having percentage inhibitions of 66.91 ± 1.533, 68.504 ± 0.000 and 23.077 ± 6.654 % at 0.05 mM respectively. At 0.8 mM, zinc totally inhibited dehydrogenase activity in *Bacillus sp.* DISK1 and *Escherichia coli*. Total inhibition of dehydrogenase activity occurred at higher zinc concentration of 1.6 mM in *Pseudomonas sp.* DAF1. In all the bacteria except *Pseudomonas sp.* RWW2, total inhibition of dehydrogenase activity by cadmium occurred at either 0.6 or 0.8 mM. In *Pseudomonas sp.* DAF1, cobalt totally inhibited dehydrogenase activity at 1.4 mM. Cobalt inhibited dehydrogenase activity by 96.752 ± 0.703 and 88.846 ± 1.692 in *Bacillus species* and *Escherichia coli* respectively.

The inhibitory effects of the metal ions on dehydrogenase activity are consistent with reported toxic effects of metals on microbial metabolic processes (Ji and Silver, 1995; Nies, 1999; Coello Oviedo *et al*., 2002; Wang and Crowley, 2005). Although some heavy metals (e.g. Cu, Fe, Zn, Co, Mn and Ni) are trace elements and are essential at low concentrations for normal cellular metabolism, they are toxic at high concentrations. For instance, zinc ion concentrations at 0.0001-0.01 mM are required for optimal growth in most microrganisms (Sugarman, 1983). However, at concentration beyond physiologically required level, zinc inhibits respiratory activities in microorganisms (Kleiner, 1978; Kasahara and Anraku 1974; Pérez-Garcia *et al*., 1993; Beard *et al*., 1995). Cobalt occurs in co-factor B_{12} and nitrile hydratases (Nies, 1999; Kobayashi and Shimizu, 1998). At high concentrations, cobalt interacts with Fe^{2+} and inhibits its physiological function (Nies, 1999). On the other hand, cadmium is a non-essential metal and is toxic to living cells even at very low concentrations. Cd^{2+} displaces Ca^{2+} or Zn^{2+} in

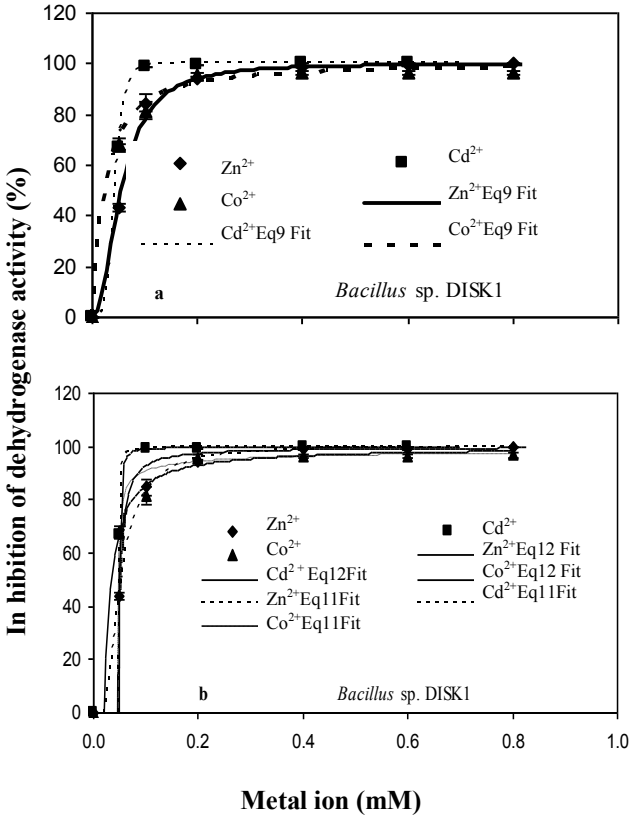

Figure 4: Effects of metals on dehydrogenase activity in *Bacillus* sp. DISK1. Experimental data mean ± 1SD (n = 3) as data points and bars are shown with predicted values derived from equation 9 (a) and equations 11 and 12 (b).

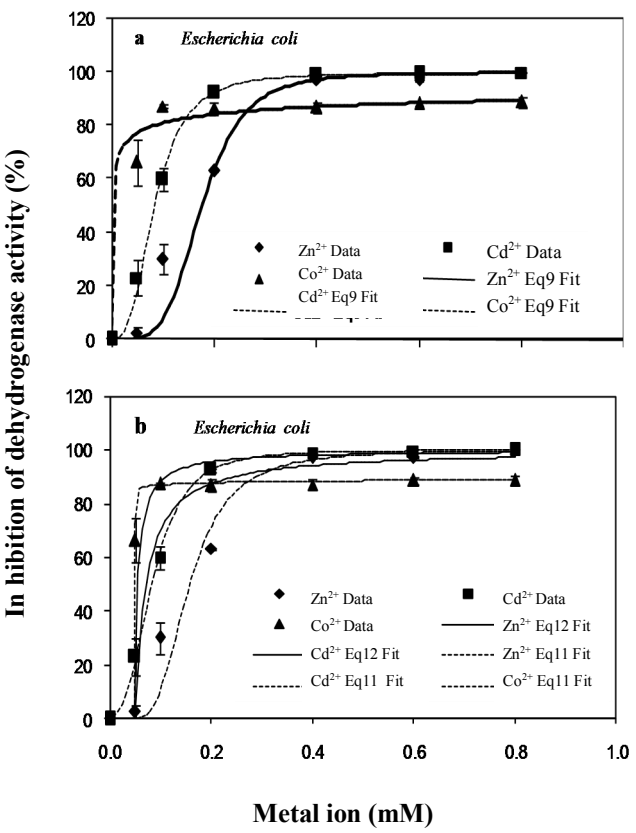

Figure 5: Effects of metals on dehydrogenase activity in Escherichia coli. Experimental data mean ± 1SD (n = 3) as data points and bars are shown with predicted values derived from equation 9 (a) and equations 11 and 12 (b).

proteins and can cause oxidative stress (Stohs and Bagchi, 1995; Goyer, 1997). Zn^{2+}, Cd^{2+} and Co^{2+} were reported to inhibit the growth of *Pseudomonas aeruginosa, Bacillus thuringiensis and Escherichia coli* K12 (Hassen *et al*., 1998a,b). Although the mechanism of the inhibition of dehydrogenase activity was not investigated in this study, metals have been reported to disrupt integrity of cell membrane (Gadd, 1993) which is the site of dehydrogenase activity in bacteria. Cd^{2+} was able to alter the outer membranes of bacteria and disturb the proton flux through the membrane (Bitton *et al*., 1988). However, Surowitz *et al.,* (1984) showed that the effects of Cd^{2+} on respiration of sensitive strains seemed to involve metabolic mechanisms prior to the entry of electrons in the electron transport system rather than on the transport system itself.

The effect of metal ions on the dehydrogenase activity in the bacterial strains can be described mathematically with kinetic logistic functions and in the form similar to non-competitive inhibition of enzyme activity. Equation 9 was used to determine the inhibition coefficient (K_i) and predict the toxicity threshold concentrations (IC_{20}, IC_{50} and IC_{80}). The toxicity threshold concentration *a*, which is the concentration of metal ions above which there is inhibition, was predicted from equations 11 and 12. The values of ICs, K_i and *a* are shown in Tables 1 and 2. *Bacillus sp.* DISK1, *Pseudomonas sp.* DAF1 and *Escherichia coli* are most sensitive to the toxicity of Zn^{2+}, Cd^{2+} and Co^{2+} respectively. Based on the IC_{50}, the order of sensitivity is *Bacillus sp*. DISK1 > *Escherichia coli* > *Pseudomonas sp*. DAF1 > *Pseudomonas sp*. RWW2 (for Zn^{2+}), *Pseudomonas sp*. DAF1 > *Bacillus sp*. DISK1 > *Escherichia coli* > *Pseudomonas sp*. RWW2 (for Cd^{2+})

Table 1: Parameter estimates from heavy metal inhibition of dehydrogenase activity data.

Bacteria[a]	Metal	Equation 9			Equation 11				Equation 12			
		KI	K_i	R^2 adj	*a*	*KI*	K_i	R^2 adj	*a*	K_i	R^2 adj
DAF1	Zn^{2+}	1.023	0.3798	0.987	0.0452	0.791	0.3886	0.997	0.0085	0.3731	0.987
	Cd^{2+}	1.307	0.0093	0.999	0.00001	1.309	0.0093	0.999	0.0183	0.0147	0.982
	Co^{2+}	0.433	0.3286	0.990	9.588×10^{13}	0.461	0.3072	0.988	-0.2489	0.3461	0.918
RWW2	Zn^{2+}	0.887	0.6265	0.996	0.0013	0.882	0.6263	0.988	-0.0251	0.6273	0.982
	Cd^{2+}	1.279	0.2149	0.990	0.0445	1.109	0.2271	0.980	0.0490	0.2293	0.963
	Co^{2+}	0.874	0.3263	0.998	0.0245	0.728	0.3270	0.993	-0.0215	0.3251	0.988
DISK1	Zn^{2+}	2.119	0.0022	0.999	0.0228	1.716	0.0026	0.997	0.0468	0.0041	0.977
	Cd^{2+}	5.101	1.1382×10^{-7}	0.999	0.0494	0.900	0.0007	0.999	0.0490	0.0005	0.999
	Co^{2+}	1.175	0.0125	0.996	0.0471	0.507	0.0236	0.841	0.0206	0.0125	0.828
E. coli	Zn^{2+}	4.396	0.0005	0.991	0.0500	2.197	0.0028	0.957	0.0500	0.0220	0.051
	Cd^{2+}	2.881	0.0008	0.999	-0.0147	3.182	0.0006	0.995	0.0481	0.0071	0.517
	Co^{2+}	0.334	0.1083	0.999	0.0500	0.067	0.1232	0.547	-	-	-

[a] *Pseudomonas sp.* DAF1, *Pseudomonas sp.* RWW2, *Bacillus sp.* DISK1 and *Escherichia coli*

and *Escherichia coli* > *Bacillus sp.* DISK1 > *Pseudomonas sp.* DAF1 > *Pseudomonas sp*. RWW2 (for Co^{2+}). Cd^{2+} (as $CdCl_2$) was reported to inhibit dehydrogenase activity in sediment bacteria by 20 % at 60 ppm (0.327 mM). The inhibitory effect of Cd^{2+} on *Bacillus* species was detected at 0.05 mM. This inhibition was marked at 0.10 mM and lethal at 0.15 mM (Montuelle *et al*., 1994). According to Kleiner (1978), Zn^{2+}, Cd^{2+} and Co^{2+} inhibited the oxidation of NADH by 50% at 0.003, 0.004 and 0.025 mM respectively. Similarly, oxidation of NADPH and succinate was inhibited by 50 % at 0.008 and 0.012 mM (Zn^{2+}), 0.025 and 0.012 mM (Cd^{2+}) and 0.1 and 0.4 mM (Co^{2+}) (Kleiner, 1978). On the basis of respiration inhibition in *Pseudomonas fluorescens*, EC_{50}s of $CdCl_2.2.5H_2O$ and $ZnCl_2$ were 52.6 mg/l (0.230 mM) and 74.3 mg/l (0.545 mM) respectively (Codina *et al*., 1993). In some cases, the toxicity threshold reported in this study corroborates other reports in the literature. Variations in the toxicity threshold are attributable to a number of factors including the genetics of the bacteria and the assay conditions.

The reciprocal of the inhibition coefficient represents the affinity of the enzyme to the toxicants. Small K_i indicates strong affinity between the enzyme and the toxicant, and thus the more strongly the enzyme is inhibited at a given concentration of toxicant. Therefore, smaller K_i means higher toxicity and smaller IC_{50}. The K_i estimates were compared with IC_{50}s obtained with each metal ion. The Pearson product-moment correlation coefficient (r) is calculated as 0.9802 for Zn^{2+}, 0.9834 for Cd^{2+} and 0.7122 for Co^{2+}. Based on K_i, the order of sensitivity is *Escherichia coli* > *Bacillus sp*. DISK1 > *Pseudomonas sp*. DAF1 > *Pseudomonas* RWW2 for Zn^{2+}, *Bacillus sp.* DISK1 > *Escherichia coli* > *Pseudomonas sp.* DAF1 > *Pseudomonas sp.* RWW2 for Cd^{2+} and *Bacillus sp.* DISK1 > *Escherichia coli* > *Pseudomonas* sp. RWW2 > *Pseudomonas sp.* DAF1 for Co^{2+}. This sequence is

Table 2: Inhibition threshold concentrations of metals

Bacteria	Metal	Toxicity thresholds (mM)		
		IC_{20}	IC_{50}	IC_{80}
Pseudomonas sp. DAF1	Zn^{2+}	0.113 ± 0.018	0.397 ± 0.046	1.392 ± 0.104
	Cd^{2+}	0.010 ± 0.002	0.028 ± 0.002	0.081 ± 0.004
	Co^{2+}	0.004 ± 0.000	0.081 ± 0.016	1.608 ± 0.688
Pseudomonas sp. RWW2	Zn^{2+}	0.137 ± 0.035	0.634 ± 0.095	2.949 ± 0.140
	Cd^{2+}	0.096 ± 0.016	0.302 ± 0.038	0.955 ± 0.084
	Co^{2+}	0.061 ± 0.021	0.278 ± 0.069	1.286 ± 0.192
Bacillus sp. DISK1	Zn^{2+}	0.032 ± 0.001	0.055 ± 0.002	0.092 ± 0.007
	Cd^{2+}	0.033 ± 0.001	0.043 ± 0.001	0.057 ± 0.001
	Co^{2+}	0.009 ± 0.000	0.028 ± 0.001	0.084 ± 0.010
Escherichia coli	Zn^{2+}	0.119 ± 0.007	0.172 ± 0.004	0.249 ± 0.003
	Cd^{2+}	0.048 ± 0.007	0.083 ± 0.008	0.142 ± 0.006
	Co^{2+}	0.001± 0.001	0.009 ± 0.007	0.113 ± 0.050

similar to that based on IC_{50}. Ren and Frymier (2003) reported similar agreement between K_i and IC_{50} obtained from bioluminescence inhibition data. This indicates that K_i can be used as a measure of toxicity. It can be seen from equations 8 and 9 that K_i is actually a function of IC_{50} and is related as $K_i = IC_{50}^{KI}$ Similarly, the K_i estimates obtained from equations 9, 11 and 12 were compared. The Pearson product-moment correlation coefficient (r) for equation 9 versus equation 11 comparison were 0.9999 (Zn^{2+}), 0.9998 (Cd^{2+}) and 0.9995 (Co^{2+}). In equation 9 versus equation 12 comparison, r was 0.9998 (Zn^{2+}), 0.9997 (Cd^{2+}) and 0.9987 (Co^{2+}). The 2-way ANOVA results showed that the dehydrogenase activity varied significantly ($p < 0.05$) with bacterial type and the concentration of metal ions.

The toxicity threshold concentration a, represent the maximum concentration of the metal ion required for normal physiological activity of bacteria. Although the minimum concentrations of Zn^{2+}, Cd^{2+} and Co^{2+} that would inhibit dehydrogenase activity in the bacterial strains were not determined in this work, it would be the concentration that caused 100% inhibition. Based on non competitive inhibition of dehydrogenase activity (equation 12), toxicity threshold a of Zn^{2+} was reported as 0.145mM and 0.099 mM respectively for *Pseudomonas sp.* PLK5 and *Escherichia sp.* PLK1 isolated from river water (Nweke, 2009). While equation 12 predicts negative inhibition of dehydrogenase activity for $I < a$, inhibition of dehydrogenase activity for $I < a$ is undefined with equation 11. However, equation 12 was not used to predict the effect of metal ions at concentrations below a. In some organisms, the actual threshold concentrations were not detected. However, the models returned negative values. This is attributed to high percentage inhibition of dehydrogenase activity at low concentration (0.05 mM) of the respective metals indicating that relatively lower concentrations of the metal ions are required to inhibit dehydrogenase activity in the organisms. Thus, it means that the threshold metal ion concentration (a) would be below 0.05 mM. Below a, stimulation of dehydrogenase activity is expected. Such stimulation was observed in *Pseudomonas sp.* RWW2 at 0.05 mM Cd^{2+} where dehydrogenase activity was stimulated by 6.613 ± 4.675%. The reason for this stimulation is not known, as cadmium is not known for any physiological function.

The results of this study have shown that zinc, cadmium and cobalt are toxic to bacterial metabolic activities. Degradation of organic matter is a dehydrogenation process. Therefore, inhibition of dehydrogenase enzyme activity would result to the inhibition of biodegradation activities. The

concentrations of metal ions in wastewater needed to be finely adjusted to prevent metal deprivation or toxicity. In this regard, the concept of threshold concentrations of toxicant above which toxic effect is observed is a valuable information. It represents the maximum concentration of toxicant required for normal physiological activities of the bacteria. The dynamics of the toxic action could be described by logistic function and in the form similar to non-competitive inhibition of enzymes. The information obtained from the models could be useful in the design and operation of industrial wastewater system as well as formulation of discharge limits.

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