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Impacts of some divalent cations on periplasmic nitrate reductase and dehydrogenase enzymes of *Escherichia, Pseudomonas and Acinetobacter* species

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ABSTRACT

The impacts of Hg^{2+}, Cd^{2+} and Zn^{2+} on the activities of periplasmic nitrate reductase (NAP) and dehydrogenase (DHA) enzymes of three organisms isolated from soil and sediment-water interface were analysed in liquid culture studies. NAP and DHA activities were estimated from nitrite and triphenyl formazan produced respectively after 4h incubation at 28 ± 2°C. Hg^{2+} completely inhibited NAP activity in *Escherichia and Pseudomonas* spp. at all the concentrations (0.2 – 1mM) while progressive inhibitions of NAP activity were observed in *Escherichia and Pseudomonas* spp. with increasing concentrations of Zn^{2+} and Cd^{2+}. Both metals were stimulatory to NAP of *Acinetobacter* sp. at 0.2 – 1mM. Apart from stimulation of DHA activity by Zn^{2+} (0.2 – 1mM) in *Escherichia* sp., Cd^{2+} (0.4 -1.0mM) in *Acinetobacter* sp. and (1.0mM) in *Pseudomonas* sp., all the metals progressively inhibited DHA activities in the three organisms. In *Escherichia* sp., the activities of the two enzymes were negatively correlated on exposure to Zn^{2+} (r = -0.91) and positively correlated (r = >0.90) on exposure to Cd^{2+} and Hg^{2+}. Based on IC_{50} values of the metals for the DHA and NAP enzymes, the most resistant of the three organisms were *Escherichia* sp. and *Acinetobacter* sp. respectively. Quantitatively, NAP with its lower IC_{50} values than DHA was a more sensitive toxicity measure for Hg^{2+} in all the organisms. The sensitivity of microbial metabolic enzymes to the toxic effects of metals varies with the type of enzyme, metal and the microorganism involved.

Keywords: Periplasmic nitrate reductase; Dehydrogenase; *Escherichia* sp.; *Pseudomonas* sp.; *Acinetobacter* sp.; IC_{50}; Hg^{2+}; Cd^{2+} and Zn^{2+}.

Impactos de alguns cátions divalentes em reductase de nitrato periplásmico e dehydrogenase de enzimas das espécies *Escherichia, Pseudomonas e de Acinetobactérias*

RESUMO

Os impactos do Hg^{2+}, Cd^{2+} e Zn^{2+} nas atividades de reductase do nitrato periplásmico (NAP) e dehidrogenase (DHA) de enzimas em três organismos isolados do solo e da interface sedimento-água foram analisados em estudos de cultura líquida. As atividades de NAP e DHA foram estimadas com o uso de formazan de trifenil formado depois de 4h de incubação a 28 ± 2°C. O Hg^{2+} inibiu a atividade da NAP completamente em *Escherichia* e *Pseudomonas*

spp em todas as concentrações (0,2 – 1 mM) enquanto foram observadas inibições progressivas da atividade da NAP em *Escherichia* e *Pseudomonas* spp com concentrações crescentes de Zn^{2+} e Cd^{2+}. Ambos os metais foram estimuladores da NAP em *Acinetobactéria* sp para 0.2 – 1 mM. Exceto pelo estímulo da atividade DHA pelo Zn^{2+} (0.2 – 1 mM) em *Escherichia* sp, Cd^{2+} (0.4 -1.0 mM) em *Acinetobacter* sp e (1.0 mM) em *Pseudomonas* sp, todos os metais progressivamente inibiram atividades de DHA nos três organismos. Em *Escherichia* sp, as atividades das duas enzimas foram negativamente correlacionadas quando em exposição ao Zn^{2+} (r = -0.91) e positivamente correlacionadas (r = >0.90) quando em exposição ao Cd^{2+} e Hg^{2+}. Baseado em valores de IC_{50} dos metais para a DHA e enzimas NAP, os mais resistentes dos três organismos foram a *Escherichia* sp e *Acinetobactéria* SP, respectivamente. Quantitativamente, a NAP com seu valor mais baixo de IC_{50} do que a DHA foi uma medida de toxicidade mais sensível para Hg^{2+} em todos os organismos. A sensibilidade de enzimas metabólicas microbianas aos efeitos tóxicos de metais varia com o tipo de enzima, metal e com o microorganismo envolvido.

Palavras-chave: Reductase do nitrato periplásmico; dehidrogenase; *Escherichia* sp; *Pseudomonas* sp; de *Acinetobactéria* sp; IC_{50}; Hg^{2+}; Cd^{2}+ e Zn^{2+}.

1. INTRODUCTION

Denitrification, the conversion of nitrate to its reduced form plays a key role in the nitrogen cycle and has important agricultural, environmental, and public health significance. Nitrate loss from agricultural soil reduces bioavailable nitrogen, hence affects crop yield as well as soil fauna and flora. Denitrification therefore is not a desirable process from an agricultural point of view (Loreau et al., 2001). However in sewage treatment where one of the goals is reduction in nitrate level of wastes before final disposal, denitrification is a desirable process. Various species of *Achromobacter*, *Agrobacterium*, *Hyphomicrobium*, *Escherichia*, *Pseudomonas*, *Vibrio* and others are responsible for denitrification in soil (Otlanabo, 1993). In wastewater treatment plants, a vast array of microbial species which includes *Aeromonas*, *Klebsiella*, *Enterobacter*, *Commomonas* and *Bacillus* have been isolated (Lim et al., 2005). Originally thought to be an entirely anaerobic microbial process, denitrification has been found to also occur under aerobic conditions. The enzyme responsible for the aerobic process is a dissimilatory nitrate reductase (NAP) which exists in the periplasm of some Gram-negative bacteria (Ellington et al., 2002; Potter et al., 2001). Isolations from soil and wastewater indicate that aerobic denitrification is widespread in nature. Despite this, the physiological role and the ecological implications of the process are still being elucidated. It was suggested that the roles of the enzyme vary in different organisms and even in the same organism under different metabolic conditions (Moreno-Vivian et al., 1999). Some of the proposed roles for the process are the disposal of excess reducing equivalents during aerobic growth and nitrate respiration in nitrate-limited environments (Ellington et al., 2002).

Dehydrogenase activity represents the intracellular flux of electrons to oxygen and is due to the activities of several intracellular enzymes catalyzing the transfer of hydrogen and electron from one compound to another (Nannipieri et al., 1990). Its measurement has been used to assess the toxicity of environmental pollutants to microorganisms (Nweke et al., 2006, 2007; Adam and Ducan, 2001). Heavy metal ions such as Hg^{2+}, Cd^{2+}, Zn^{2+}, Pb^{2+} and other trace metals enter the soil from both natural and anthropogenic sources. These ions have

great ecological significance due to their toxicity and accumulative behavior (Purves, 1985). The interaction between microbes and metals in the environment has been both beneficial and detrimental. Though some have been found essential for many microbial processes, at high concentrations, both essential and non essential metals are known to be toxic to microorganisms (Nies, 1999).

The dehydrogenase enzymes from different microorganisms have been reported to respond differently to environmental stress. Ohnesorge and Wilhem (1991), observed that dehydrogenase activity in *Pseudomonas* species reduced with increasing concentrations of Cd^{2+}, while in *Proteus* species, stimulated activity was observed at Cd^{2+} concentrations of 0.2mM to 0.4mM followed by progressive inhibition at concentrations above 0.6mM. Nweke et al. (2006) also observed that Zn^{2+} at 0.2 mM stimulated dehydrogenase enzyme of *Proteus* sp PLK2 and *Micrococcus* sp. PLK4 followed by progressive inhibition thereafter, while in *Escherichia sp* PLK1 and *Pseudomonas* sp PLK5 progressive inhibitions were observed at all the concentrations of Zn^{2+} studied (0.2-1.2mM). Unlike the dehydrogenase activity, information on the sensitivities of periplasmic nitrate reductase to environmental stress is scarce. However, Bursakov et al., (1997) reported the inhibition of the enzyme in *Desulfovibrio desulfuricans* ATCC 27774 by divalent cations. In a previous study, Okolo et al. (2007) reported that the periplasmic nitrate reductase of *Acinetobacter* sp isolated from an agricultural soil was more sensitive to phenolic compounds than the dehydrogenase enzyme of the same organism. For an enzyme such as periplasmic nitrate reductase whose desirability of inhibition or stimulation of activity depends on the specific environment under review, it becomes necessary to investigate the environmental effects of a wide array of stress factors in order to make useful decisions.

In this study, the response to metal exposure of the periplasmic nitrate reductase and dehydrogenase enzymes of organisms isolated from soil and sediment-water interface, which represent two different environments in which nitrate reduction plays different environmental roles is reported.

2. MATERIALS AND METHODS

2.1. Isolation, purification and screening of organisms for nitrate reduction

Samples were collected randomly from an agricultural farm at a depth of 5 cm below the soil surface and from the upper 5cm of sediment-water interface of Nwaorie River using a sterile metal cylindrical tool. Samples treatment, media and incubation conditions were as previously described (Okolo et al., 2007). Purification and screening for nitrate reduction was done in a defined medium of Celen and Kilic (2004), as modified by Okolo et al. (2007). The modified defined agar medium has the following composition (g/litre): succinic acid, 3.54; NaOH, 1.2; NH_4Cl, 0.535; Na_2HPO_4,18; KH_2PO_4,1.0; NaCl, 2.5; $MgSO_4$, 0.1; $FeSO_4$, 1.11; fungicide (Ketoconazole), 0.05; agar 17; pH 7.2.

The selected organisms were stored on slants of the defined medium prior to characterisation up to the generic level following the schemes of Holt et al. (1994).

2.2. Preparation of inoculum

The organisms were plated on the modified defined medium (Okolo et al., 2007) containing 10 mM KNO_3 instead of the 10 mM NH_4Cl and incubated at room temperature (28 ± 2 ^oC) for two days. The cultures were washed, harvested and standardized by resuspending in phosphate buffered saline and adjusting the turbidity to give an optical density of 0.4 at 600

nm as previously described (Okolo et al., 2007). The cell suspensions served as the standardized inocula for the studies. The dry weights of the cells were estimated by drying a 2 ml aliquot of the standardized cell suspensions to constant weight in a pre-weighed crucible in an oven at 105^{o}C.

2.3. Assay for toxicity of metals to enzyme activities

Portions (0.2ml) of the standardized cell suspensions were inoculated into sterile triplicate 20 ml screw-capped glass tubes containing 4.8 ml of the modified defined medium supplemented with a particular concentration (0.2 – 1.0 mM) of the various metals (Zn^{2+}, Cd^{2+} and Hg^{2+}) prepared in the modified defined medium (Okolo et al., 2007) devoid of nitrate (KNO_3) and agar. The control consisted of the inoculated medium without the metals.

Incubation conditions and assays for the periplasmic nitrate reductase and dehydrogenase activities were as described earlier (Okolo et al., 2007). Nitrite concentrations and the amount of formazan produced in the samples were estimated by reference to standard dose-response curves. Periplasmic nitrate reductase (NAP) activity was expressed relative to the amount of nitrite formed while dehydrogenase (DHA) activity was expressed as milligrams of triphenyl formazan (TPF) formed per mg dry weight of cell biomass per hour.

2.4. Calculation of inhibition or stimulation of enzyme activity

Inhibitions or stimulations of the enzyme activities were calculated relative to the controls. Where applicable, the IC_{50} and IC_{20} of the metals were determined by fitting the percentage inhibition values to simple equations using Table 2D Curve (Systat Inc., USA) and calculating the concentrations of the metals at 50 % inhibition of enzyme activity.

2.5. Statistical analysis

Data generated were subjected to multiple factor analyses of variance (ANOVA). Relationships between the effects of divalent cations on enzyme activities were analyzed using regression analyses and the Pearson's product-moment correlation coefficient.

3. RESULTS AND DISCUSSION

The isolate from the sediment-water interface was identified as *Escherichia* sp while those from soil were identified as *Pseudomonas* and *Acinetobacter* spp. The three organisms are Gram negative non-spore-forming rods and members of these genera are often ubiquitous and exhibit a great deal of metabolic versatility. Members of the genus *Pseudomonas* demonstrate a great deal of metabolic diversity, and consequently are able to colonise a wide range of niches (Madigan and Martinko, *2005).* The *Acinetobacter* have been attracting growing interest in both environmental and biotechnological applications because they possess characteristics which are being exploited in various biotechnological applications including bioremediation of heavy metal contaminated waters (Boswell et al., 1999). According to Otlanabo (1993), various species of *Escherichia and Pseudomonas* among others are responsible for denitrification occurring in incredible diverse microbial consortia as that in wastewater.

The demonstration of a periplasmic nitrate reductase (NAP) activity by these organisms is proved by the experimental protocols used in the study. Nitrate reduction in bacteria is brought about by the activities of three nitrate reductases which are active under different physiological conditions. These are the cytoplasmic nitrate reductase (NAS), membrane-

bound nitrate reductase (NAR) and the periplasmic nitrate reductase (NAP). The activity of NAS is repressed by ammonium in the isolation medium while that of NAR is repressed under the aerobic conditions used in the study. NAP is not affected by ammonium or oxygen (Celen and Kilic, 2004; Simon et al., 2003).

The results of the effects of different concentrations of Zn^{2+}, Cd^{2+} and Hg^{2+} on the NAP and dehydrogenase (DHA) activities of *Escherichia*, *Acinetobacter and Pseudomonas* species are shown in Figures 1 and 2. NAP activity was most prolific in *Acinetobacter* sp., yielding 2.19µgNO_2-N/ml in the control treatment (Figure 1) while DHA activities were comparable in all the organisms. In all the organisms, differences in the responsiveness of NAP and DHA to increasing concentrations of the three metals were significant ($P < 0.5$). Except Hg^{2+} which completely inhibited NAP activities in *Escherichia and Pseudomonas* spp at all the concentrations, responses of the enzymes to the metals were dose-dependent. Increasing concentrations of Zn^{2+} and Cd^{2+} resulted to progressive reductions in nitrite production in *Escherichia and Pseudomonas* spp. (Figure 1). In *Acinetobacter* sp., sharp increases in NAP activities were observed on exposure to Zn^{2+} and Cd^{2+} at 0.2mM, thereafter, progressive reduction in activities followed (Figure 1). High toxicity of Hg^{2+} reported in various microbes has been attributed to its very high affinity to thiol groups (Nies, 1999). This has tremendous relevance in this study because the molecular structures of NAP show that cysteine provides a thiol ligand to the molybdenum cofactor at the active sites of the enzyme (Jepson et al., 2004). In a similar study, Bursakov et al. (1997) reported the inhibition of NAP activities in *Desulfovibrio desulfuricans* ATCC 27774 by divalent cations**.** The increase in NAP activity of *Acinetobacter* sp at Cd^{2+} (0.2-1.0mM) indicates that Cd^{2+} at the stated concentrations might be a cofactor for the enzyme in the organism. Apart from stimulation of DHA by Zn^{2+} (0.2 – 1mM) in *Escherichia* sp, by Cd^{2+} (0.4 -1.0mM) in *Acinetobacter* sp., and by Cd^{2+} (1.0mM) in *Pseudomonas* sp, all the metals inhibited DHA activities at various degrees in the three organisms (Figure 2). Nweke et al. (2006) reported that Zn^{2+} at 0.2 mM stimulated DHA of *Proteus* sp PLK2 and *Micrococcus* sp. PLK4 isolated from a river water sample and thereafter progressive inhibition followed at higher concentrations. In the same study, however, Nweke et al. (2006) noted progressive inhibition of DHA activities in *Escherichia* sp PLK1 and *Pseudomonas* sp PLK5 at all the concentrations of Zn^{2+} studied (0.2-1.2mM). These differences in the responsiveness of DHA enzymes from different organisms exposed to Zn^{2+} could be attributed to variations in cell wall components of microorganisms and might also be related to genetic factors of metal resistance among the organisms. Although Zn^{2+} is an essential trace element that plays vital role in cell growth, differentiation and development, (Ohnesorge and Wilhelm, 1991), it is known to be a potent inhibitor of the respiratory electron transport system (Beard et al., 1995).

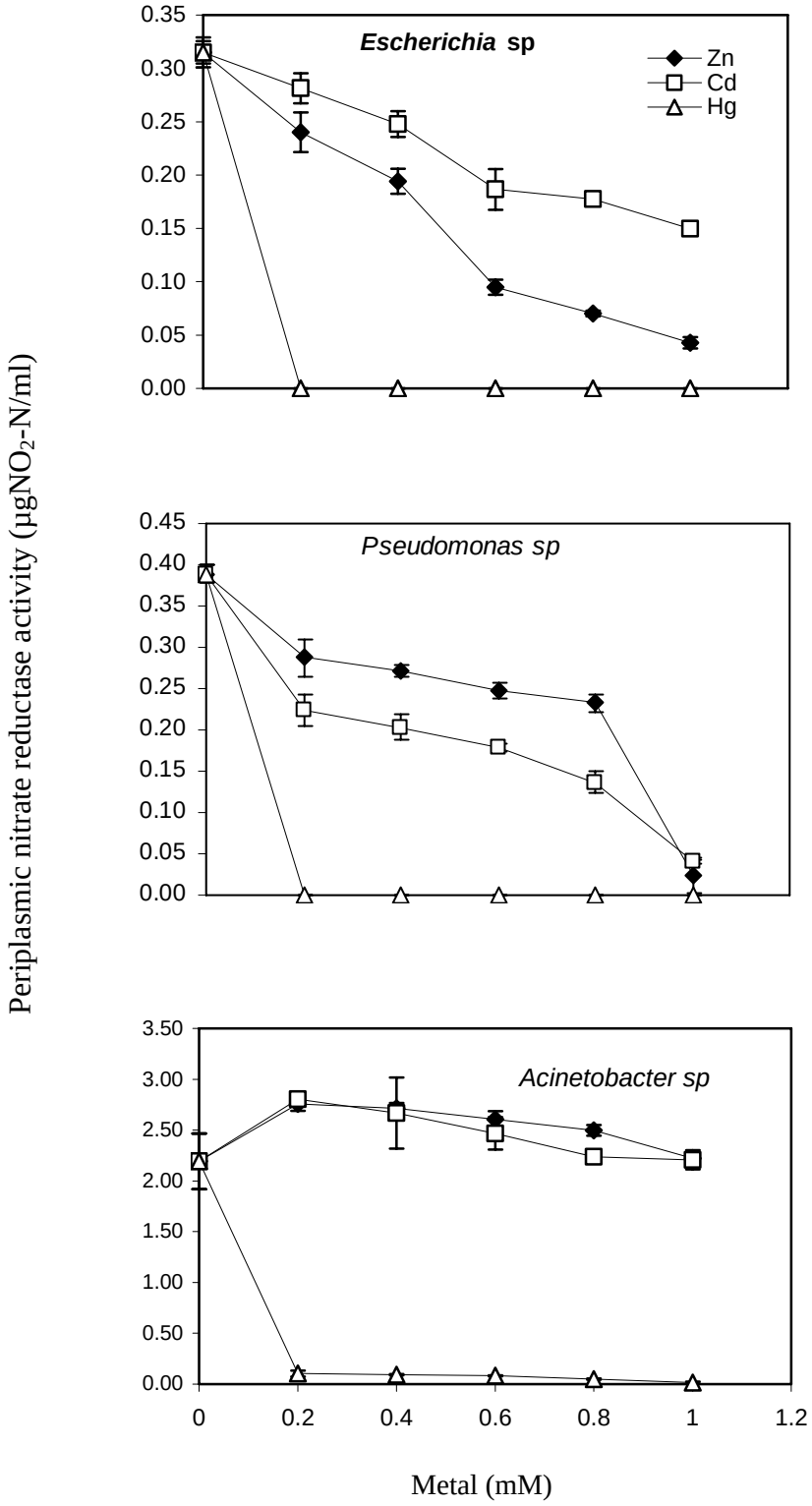

Figure 1. Effects of metals on periplasmic nitrate reductase activities of the bacterial strains.

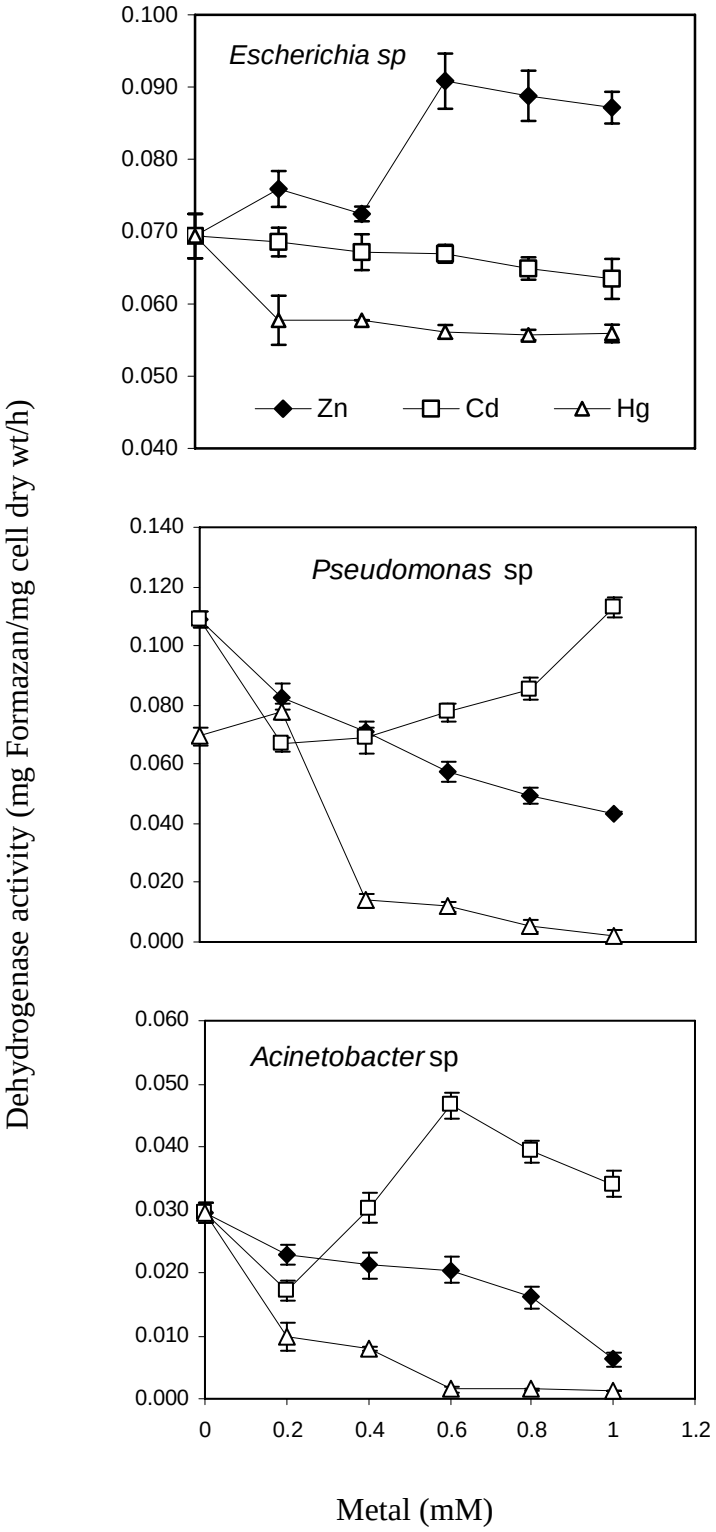

Figure 2. Effects of metals on dehydrogenase activities of the bacterial strains.

With respect to increasing concentrations of Cd^{2+}, the response of the DHA enzymes of the organisms varied markedly. In *Escherichia* sp., slight reductions in formazan production were observed as Cd^{2+} concentrations increased (Figure 2). On the contrary, in *Pseudomonas* sp., lower concentrations of Cd^{2+} inhibited formazan production while increased production was observed at 1.0mM Cd^{2+} (Figure 2). In *Acinetobacter* sp., an initial decrease in formazan production was observed at a Cd^{2+} concentration of 0.2 mM, thereafter, formazan production increased with increasing concentrations of Cd^{2+} up to 0.6mM. Above 0.6mM, a further increase in Cd^{2+} concentrations led to a decrease in formazan production (Figure 2). The stimulatory effects of Cd^{2+} indicate that it could be a necessary cofactor for the DHA of the *Pseudomonas* sp at 1.0mM and for the *Acinetobacter* sp. at 0.6mM. This report is however contrary to the observations of Liao et al. (2005), in which dehydrogenase activity decreased with increasing Cd^{2+} concentrations. These variations in responses of the DHA enzymes from different microorganisms exposed to metal ions could be accounted for by the fact that dehydrogenase systems differ in organisms (Praveen-Kumar, 2003).

The associations between the activities of NAP and DHA enzymes of the three organisms were estimated using the Pearson's product moment correlation coefficient. Results indicated that negative correlations existed between the two enzymes on exposure of *Escherichia* sp. to Zn^{2+} ($r = -0.91$) and of *Pseudomonas* and *Acinetobacter* spp. to Cd^{2+} ($r = -0.01$ and -0.49 respectively). Apart from these, associations between the two enzymes in the three organisms in response to increasing concentrations of the metals were positively correlated. This positive correlation was strong (>0.9) in *Escherichia* sp in response to Cd^{2+} and Hg^{2+}, and in *Acinetobacter* sp. in response to Hg^{2+}. In a similar study, Okolo et al. (2007), reported a positive correlation between NAP and DHA enzymes of *Acinetobacter* sp. exposed to various doses of phenolic compounds and attributed it to the fact that both enzymes are membrane associated. Metals are also known to have effects which directly or indirectly disrupt microbial membranes. It has been suggested that Zn^{2+} binds to the membranes of microorganisms, and both organic and inorganic mercurials interfere with membrane permeability and enzyme reactions through binding to sulfhydryl groups (Eisler, 2006) and forming nonspecific intracellular complexes with thiol groups (Nies, 1999). Since the microbial process being investigated is aerobic nitrate reduction, interactions of these nonspecific complexes with molecular oxygen could lead to the formation of reactive oxygen species resulting in oxidative stress within the organisms (Kachur et al., 1998). Furthermore, the three organisms under study are Gram-negative organisms and it has been reported that in Gram-negative bacteria, heavy metal cations can bind to glutathione, a notable antioxidant, resulting in considerable oxidative stress (Kachur et al., 1998; Nies, 1999). The strong negative correlation of both enzymes in *Escherichia* sp. exposed to Zn^{2+} meant that though the enzymes were membrane-associated, the toxic effect of the metal on one of the enzymes might not be membrane related. Since NAP is active in the periplasmic membrane while DHA enzyme is active intracellularly, it appears that for *Escherichia* sp., Zn^{2+} is toxic only in the periplasmic membrane.

The relative inhibitory/stimulatory effects of the different concentrations of the metals on NAP and DHA activities of *Escherichia, Pseudomonas and Acinetobacter* spp. are shown in Figures 3 and 4 respectively. Hg^{2+} at all the concentrations studied, gave 100% inhibition of NAP activities in *Escherichia* and *Pseudomonas* spp. and >90% inhibition in *Acinetobacter* sp. (Figure 3). The inhibition ranges of NAP in response to increasing concentrations of Zn^{2+} were 22.38 - 83.81% for *Escherichia* sp.; 24.81 – 91.47 % for *Pseudomonas* sp.; and for the *Acinetobacter* sp., Zn^{2+} was stimulatory. For the DHA enzyme, Zn^{2+} was stimulatory to

Escherichia sp. at all the concentrations while Cd^{2+} was also stimulatory to *Acinetobacter* sp. above 0.2mM (Figure 4). The inhibitory threshold concentrations (Table 1) of the metals for the NAP and DHA enzymes were calculated from the percentage inhibition values versus toxicant concentration plots (not shown) by fitting data into simple equations. Readings are taken from regression curves with high coefficients of determination ($0.9 \leq R^2 \leq 1.0$). The inhibitory threshold concentrations for the DHA enzyme which is a measure of the general microbial activity shows that *Escherichia* sp., being stimulated by Zn^{2+} and having an IC_{50} of >1 for Hg^{2+} is the most resistant of the three organisms to the effects of these metals. Greater resistance of *Escherichia* sp might not be unrelated to the fact that the organism was isolated from the sediment-water interface of a river where hospitals, schools and industries discharge their effluents. Such effluents might contain a variety of heavy metals to which the species might have adapted. Nweke et al. (2006), reported an IC_{50} of 0.301mM for Zn^{2+} for the DHA of *Escherichia* sp. PLK1 while Pérez-Garcia et al. (1993) reported an IC_{50} of 0.999mM for Zn^{2+} for the DHA of *Pseudomonas fluorescens.* Apart from the sources of organisms, these variabilities in inhibitory threshold concentrations might not be unrelated to experimental protocols like media composition and pH which affect solution-phase metal concentrations (Nies, 1999) which were analysed in the studies cited.

With respect to NAP activity, *Acinetobacter* sp. is the most resistant, being stimulated by both $Zn^{2+}$and Cd^{2+}, and showing some degree of tolerance to some concentrations of Hg^{2+}. Quantitatively, NAP with its lower IC_{50} values (<0.2mM) was a more sensitive toxicity measure than DHA activity for Hg^{2+} in all the organisms, while DHA with lower IC_{50} values was a more sensitive toxicity measure than NAP for Zn^{2+} and Cd^{2+} in *Pseudomonas* sp. Results in other organisms are varied. In *Pseudomonas* sp., Hg^{2+} is a more sensitive metal toxicity measure of NAP activity than DHA. The implication of this therefore is that the sensitivity of microbial metabolic enzymes to the toxic effects of metals varies with the type of enzyme, metal and the microorganism involved.

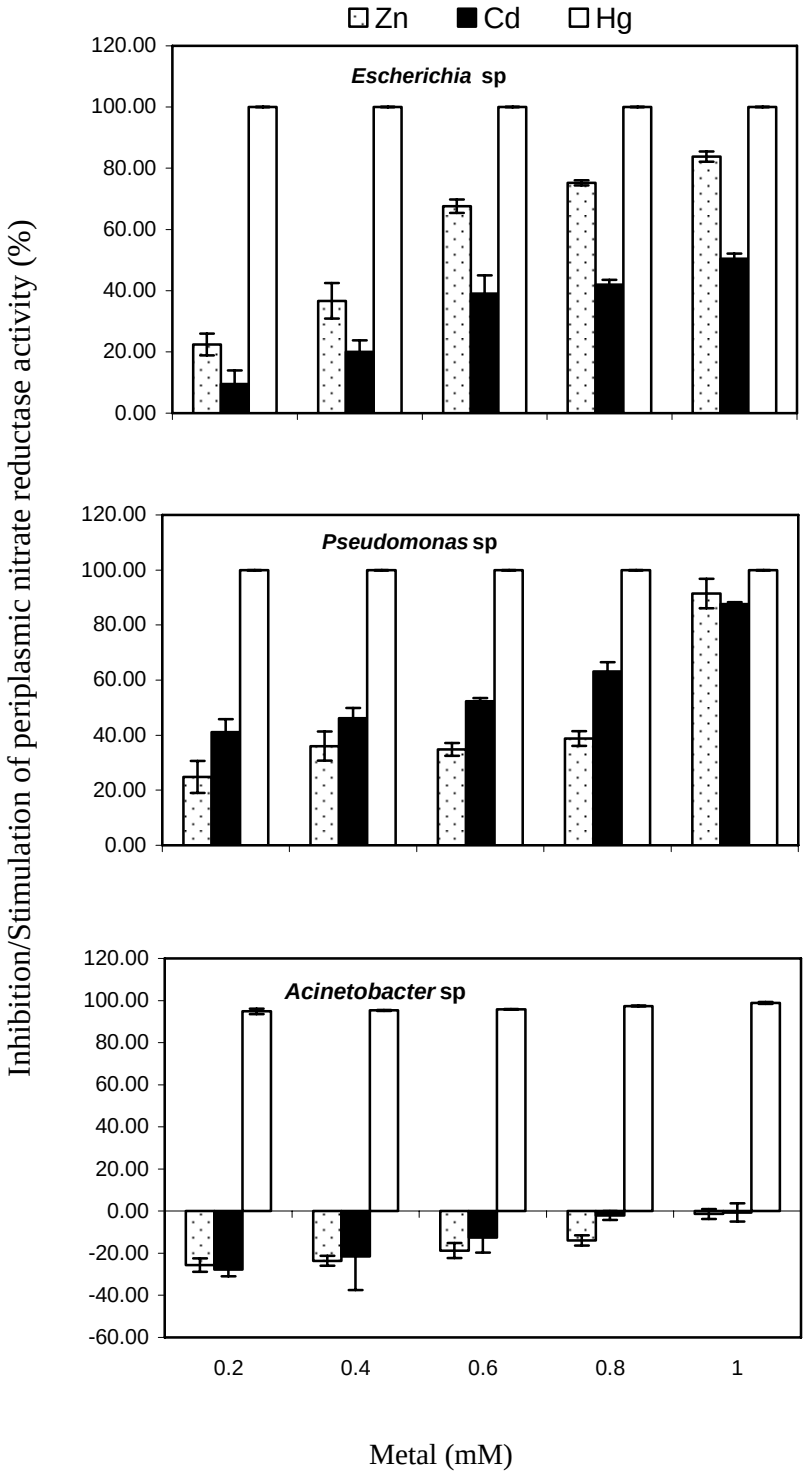

Figure 3. Relative effects of metals on periplasmic nitrate reductase activities of the bacterial strains. (> 0% = Inhibition; < 0% = Stimulation).

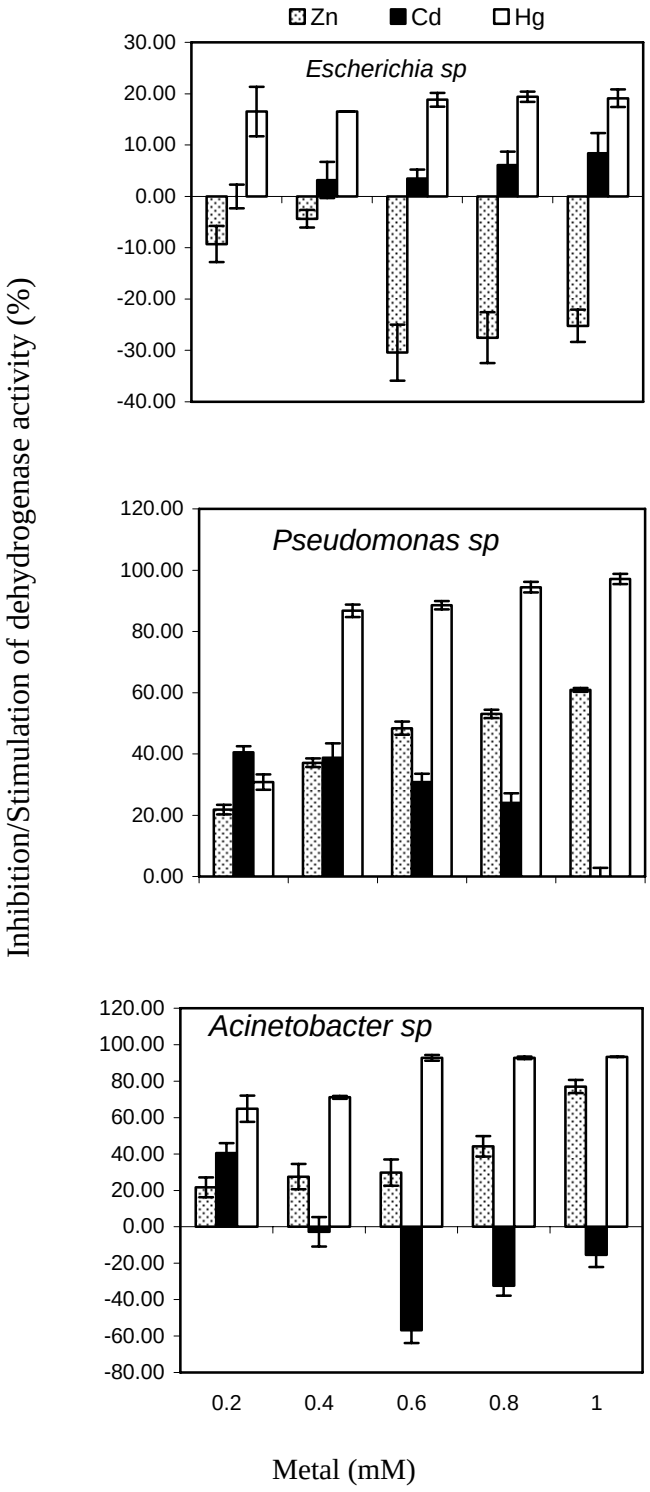

Figure 4. Relative effects of metals on dehydrogenase activities of the bacterial strains. (> 0% = Inhibition; < 0% = Stimulation).

Table 1. Threshold inhibitory concentrations of heavy metals against bacterial strains.

Bacteria	Metal	Inhibition threshold concentrations (mM)					
		Dehydrogenase activity			Periplasmic nitrate reductase		
		Model	IC_{50}	R	Model	IC_{50}	R
Pseudomonas sp.	Zinc	LDRM[a]	0.697	1.0	Poly2[a]	0.775	0.91
	Cadmium	Poly 3	< 0.2	0.99	Poly2[b]	0.71	0.98
	Mercury	LDRM	0.283	1.0	LDRM	< 0.2	1.0
Acinetobacter sp.	Zinc	LDRM	0.85	1.0	Stimulatory	–	–
	Cadmium	Stimulatory	–	–	Stimulatory	–	–
	Mercury	LDRM	0.175	0.97	LDRM	0.00165	1.0
Escherichia sp.	Zinc	Stimulatory	–	–	LDRM	0.443	0.98
	Cadmium	LDRM	>1.0	0.96	LDRM	0.961	0.98
	Mercury	LDRM	>1.0	0.99	LDRM	< 0.2	1.0

[a]Logistic Dose Response Model, $y = a/1+(x/b)^c$

Polynomial 3: $Y = -133.79x3 + 158.91x2 - 77.545x + 49.174$

Polynomial 2[a]: $Y= 173.99X^2 - 134.43X + 49.74$

Polynomial 2[b]: $Y= 103.69X^2 - 56.68X + 38.06$

Y= Inhibition (%); X= Metal concentration (mM); a, b and c are model parameters

4. CONCLUSIONS

Findings from this study further lends credence to the earlier observations of Okolo et al., (2007) that toxic effects of chemicals on specific oxidative microbial metabolism such as aerobic denitrification are better studied using the specific enzyme involved. Furthermore, the different sensitivities of NAP and DHA enzymes from different organisms to environmental stress are indications that generalizations on the impacts of stress factors on microbial metabolic enzymes need to be made with caution.

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