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Combined effects of metals and chlorophenols on dehydrogenase activity of bacterial consortium

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Abstract

Toxicity of Zinc, Cadmium, 4-Chlorophenol (4-CP), 2,4-Dichlorophenol (2,4-DCP) and their binary and quaternary mixtures were determined based on inhibition of dehydrogenase activity of a consortium of Pseudomonas, Bacillus, Micrococcus and Staphylococcus species. The toxicity of chemicals and their mixtures were evaluated in the concentration range of 0-3mM while Cadmium and 2,4-Dichlorophenol binary mixture range was 0-1.8mM. Zinc, 4-CP and 2,4-DCP exhibited hormetic effect at low concentrations. The IC_{50} were determined using monotonic and hormesis dose-response models. The binary and quaternary mixtures of the pollutants evaluated showed progressive inhibition of the enzyme activity. The combined effects of the mixtures on the enzyme activity of the bacterial consortium were evaluated with isobolographic representation and toxic index (TI) model. The isobolographic analysis indicated additive, synergistic and antagonistic interactions for the various binary mixtures evaluated. However, the TI of most mixtures was within the range of 0.5-2.0 and are considered additive. Modulation of the toxic interactions by the components of the mixture through synergistic and antagonistic interaction of the heavy metals and phenolic compounds against the dehydrogenase activity of the bacterial consortium were possible depending on the relative amount of the components.

Keywords: Toxicity, Heavy Metals, Phenols, Dehydrogenase, Effective dose.

Introduction

The pollution with petroleum, heavy metals, xenobiotics, organic and inorganic contaminants is a growing environmental concern that harm both terrestrial and aquatic ecosystem. These pollutants pose serious life threatening environmental pollution.

Continuous industrial and agricultural activities, and the persistent nature of the metals, are worsening the issue of heavy metal contamination. However, some heavy metals stimulate growth at low concentration by serving as enzyme cofactors[1-2]. The type, speciation, and concentration of the heavy metal and type of microorganism are determinants of toxicity of metals[3-7]. Although many heavy metals are essential for microbial growth and metabolism at low concentration, they are toxic in excess of physiologically required levels[8]. Both essential and nonessential heavy metals (physiologically and non physiologically important metals), at relatively high concentrations, cause harmful effects such as loss of membrane integrity, oxidation of vital enzymes, inactivation of microbial organelles[9,10], and harmful effect on the genetic make-up of the cell by direct reaction with DNA[11].

Phenols as organic compounds are present in wastewater effluents produced from petroleum, coal gasification plants, phenolic resin industries, pharmaceutical industries etc[12,13]. They are also byproducts during bleaching of pulp with chlorine and during disinfection of water by chlorination[14,15]. Phenoxy herbicides are degraded to produce phenolic intermediates that are toxic to a variety of microorganisms. Due to its toxicity, phenol has been classified as priority pollutant by environmental protection agency in many countries[16]. Phenols are known to disrupt the physiological activities of cell membrane[17]. The toxic effects of phenols include narcosis, inhibition of growth and uncoupling of adenosine triphosphate synthesis[18]. At sufficiently high concentrations, phenol and phenolic intermediates become toxic to organisms and to species that use them as growth substrates[19].

Enzymes are referred to as markers of soil purity. Dehydrogenase enzyme is an indicator of total physiological activity of microorganism because it occur intercellularlly in all living microbial cells and it is connected with oxido-reduction processes in microorganisms[20], by transferring protons and electrons from substrates to acceptors in an electron transport system. Reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC)-an indicator by microbial dehydrogenases[21] is a measure of microbial growth or viability in the presence or absence of toxicant. Bacteria play important role in the maintenance of natural ecosystems. Determination of the effects of toxic chemicals to bacteria serves as important criteria for the environmental risk assessment of chemicals.

Toxicological studies have provided good knowledge through which rules and regulations for management of chemicals have been drawn. In a polluted environment, whether aquatic or

terrestrial, pollutants occur as mixtures and various interactions can take place during exposure of organisms to mixtures of pollutants. The interactions include additivity, synergism, or antagonism[22]. Moreover, interactions of components in a mixture might cause significant changes in the properties of its constituents[23], which has a potential threat to human health and environmental ecosystems.

Although the toxicity of metals and phenols has been widely studied, there has not been much study on the combined toxicity of metals and phenol mixtures. The few investigations on the toxicity of metal and phenol mixtures were based on microbial growth and bioluminescence[24].

This study therefore evaluates the toxicity of mixtures of heavy metals and phenols via dehydrogenase enzyme activities of bacterial consortium.

Materials and methods

Test organisms: The bacterial consortium used for the assay consisted of *Pseudomonas sp., Micrococcus sp., Bacillus sp. and Staphylococcus sp*. which were isolated from agricultural soil. Soil samples were collected randomly using a sterile metal spatula. The samples were homogenized and sieved through a sterile 2 mm mesh. The sieved sample (2.0 g) was suspended in 18 ml of sterile distilled water and was serially diluted in 0.85%^{w}/v NaCl solution. One tenth of a milliliter (0.1 ml) of the 10^{-5} dilution was inoculated onto nutrient agar and incubated for 48 h at room temperature (28 ± 2^0C). The cultures were purified and characterized using battery of biochemical tests. Pure cultures of the organism were stored on nutrient agar slants at 4^0C.

Preparation of inoculums: The isolates were grown in a nutrient broth on a rotary shaker (150 rpm) at room temperature (28 ± 2^0C). After 24 h of incubation, the cells were harvested by centrifugation at 4000 rpm for 10 min. Harvested cells were washed twice in sterile distilled water and suspended therein. The optical densities (A_{540}) of the cell suspensions were adjusted to 0.5. Equal volumes of each standardized cell suspensions were mixed together in sterilized Erlenmeyer flask to obtain the bacterial consortium for the assay.

Test chemicals: The test chemicals and reagents used for the bioassay were analytical grades. The heavy metals used in the assay were Zinc (Zn^{+2}) and Cadmium (Cd^{+2}) as $ZnSO_4.6H_2O$ and $CdSO_4.1/7H_2O$ respectively (Fluka). Phenolic compounds used were 4-Chlorophenol (4-CP) and 2,4-Diclorophenol (2,4-DCP) (Sigma-chemical Co. USA). Stock concentrations (10 mM) of the respective chemicals were used for the assay.

Design of experiment: The binary and quaternary mixtures of zinc, cadmium, 4-chlorophenol and 2,4-dichlorophenol were evaluated using fixed ratio rays experimental design. In each case, the mixture ratio is kept constant, while the total concentration of the mixture is varied to obtain a complete dose-response relationship of the mixtures. The components of each of the binary mixture ratios studied were Zn+Cd, Zn+4-CP, Zn+2,4-DCP, Cd+4-CP and Cd+2,4-DCP in the weight to weight ratios of 0:100%, 10:90%, 25:75%, 50:50%, 75:25%, 90:10%, 100:0% .The quaternary mixtures of 2,4-DCP:Cd:Zn:4-CP were evaluated in the ratios of 10%:10%:20%:60%, 5%:5%:40%:50%, 10%:15%:30%:45%, 15%:15%:45%:25% and 10%:20%:60%:10%.

Dehydrogenase activity assay: Dehydrogenase enzyme activity inhibition was ascertained using 2,3,5-triphenyltetrazolium chloride (TTC) as the artificial electron acceptor which is reduced to the red-colored triphenylformazan (TPF)[25]. The inhibition of dehydrogenase activity was done in 2ml volume of nutrient broth (pH 7) and TTC added with different concentrations of zinc, cadmium, 4-chlorophenol and 2,4-dichlorophenol or their mixtures. A 0.5 ml portion of X4-strength of nutrient broth and appropriate volumes of sterile distilled water and stock solution (10 mM) of the respective toxicants were added to each 20 ml screw capped test tube to obtain the different binary and quaternary mixture ratios or different concentrations of individual toxicants. Thereafter, 0.2 ml each of 0.1%w/v solution of TTC and bacterial consortium suspension were added into each tube. The final concentrations of the toxicants varied from 0 to 2 mM except for binary mixtures of cadmium and 2,4-dichlorophenol which varied from 0 to 0.8 mM. Controls were also prepared which consisted of the inoculated medium without the toxicants. The cultures were incubated at room temperature (28 ± 2^0C) for 24 h. After incubation, 1ml of 1%^{v}/v Triton X-100 was added into each tube and allowed to stand for 10 min. The formazan produced in each tube was extracted with 4 ml of butanol. Absorbance of the extract was determined with a spectrophotometer at 500 nm.

Response estimation: The inhibition of dehydrogenase activity at different concentrations of the toxicants (Zn^{2+}, Cd^{2+}, 4-CP and 2,4-DCP) as individual, binary and quaternary mixtures were calculated as indicated in Equation (1) below. The inhibition (%) was generated as mean and standard deviation from triplicate determinations.

$$\text{Inhibition}\ (\%) = \frac{C_A - T_A}{C_A} \times 100 \qquad (1)$$

Where: C_A is the absorbance of TPF extract in the control (without toxicants); T_A is the absorbance of TPF extract in the tests with different concentrations of the toxicants or their mixtures[25].

Determination of IC_{50}: The dose-response data were fitted with 2-parameter logistic model (Equitation 2) to obtain their respective IC_{50} which is defined as the concentrations of the toxicants that inhibited the dehydrogenase activity of the bacterial consortium by 50%.

$$\text{Inhibition}(\%) = \frac{100}{1+\left(\frac{x}{\text{IC}_{50}}\right)^{b}} \quad (2)$$

Where: x is the concentration of the toxicant, IC_{50} is the concentration that caused 50% inhibition; b is parameter determining the relative slope at IC_{50}[25].

For hormesis, i.e. stimulation of enzyme activity at low concentration of individual toxicant or the mixtures, the IC_{50} was estimated using reparameterized Brain and Cousens model[26].

$$\text{Inhibition}(\%) = 100 - \frac{100 + fx}{1+\left[1+\left\{\frac{2f\text{IC}_{50}}{100}\right\}\right]\left(\frac{x}{\text{IC}_{50}}\right)^{b}} \quad (3)$$

Where: f is the parameter describing the degree of hormetic response.

Isobolographic analysis: The estimated IC_{50}s were used in isobolographic analysis of the binary mixture toxicity to determine the combined effects of the tested chemicals. The concentration of each component at IC_{50} (C_{mix}) were calculated and used to compute the isoboles. Triplicate isoboles were generated and plotted in an isobologram[25].

Determination of toxic index: Toxic index (TI) model was used to analyze the combined effect of the mixtures. The TI values were calculated using the expression:

$$\text{TI} = \sum_{i=1}^{n} \text{TU}_i \quad (4)$$

TU_i is the toxic unit of ith component in the mixture. Each toxic unit was computed using the expression

$$\text{TU}_i = \frac{\text{Cmix}_i}{\text{IC}_{50i}} \quad (5)$$

Where: $Cmix_i$ is the concentration of the ith toxicant in the mixture and IC_{50i} is the IC_{50} of the same toxicant when tested as an individual. TI = 1 signifies additive interaction, TI > 1 signifies antagonistic interaction and TI < 1 signifies synergistic interaction[27].

Statistical analysis: All analyses were performed in three replicates. Curve fittings were done using Sigma Plot 10.0 and data were statistically analyzed using IBM SPSS Statistics 21.

Results and discussion

Toxicity of the chemicals: The observed inhibition of dehydrogenase activity and the model fits to the observed data for zinc, cadmium, 4 –CP and 2,4 DCP toxicity against the bacterial consortium are shown in Figure-1. All the single compounds except cadmium showed hormetic curves which were characterized by stimulation of enzyme activity at low concentrations and toxicity at high concentrations. Zinc and 2,4-DCP showed hormetic effect at concentration up to 0.1 mM while 4–CP showed hormetic effect at concentrations up to 0.3 mM. Relatively, 4-CP among other toxicants exhibited greater stimulatory effect on dehydrogenase activity of the bacterial consortium. At doses above the stimulatory range, the heavy metals and phenolic compounds progressively inhibited the enzyme activity.

As mixtures, the dose-response plot and the model fittings of the binary and quaternary mixtures are shown in Figures-2 to 7. The various binary mixture ratios tested, showed progressive inhibition of the dehydrogenase activity of the bacterial consortium. However, low dose stimulation (hormesis) was observed in the 90% Zn + 10% 4-CP, 25% Cd + 75% 4-CP binary mixtures at concentrations ranging from 0.05 mM to 0.1 mM. Above the hormetic concentration range, the test chemical mixtures exerted inhibitory effects on the enzyme activity of the bacterial consortium. Cadmium and 2,4-DCP mixtures showed sharp inhibitory effect with increase in concentrations of Cadmium. For the quaternary mixtures, all the ratios evaluated showed toxic effect (no hormetic response) on the dehydrogenase activity as the concentrations increases.

Toxicity thresholds: The 24 h IC_{50} of the toxicants as individuals (singles) and mixtures as well as their statistical associations are shown in Tables-1 and 2. As single compound, 4–CP with highest IC_{50} of 0.675 ± 0.008 mM was the least toxic chemical to the bacterial consortium. The order of increasing toxicity of the individual chemicals was Cd > 2,4-DCP > Zn > 4-CP.

As binary mixtures, 24 h IC_{50} obtained showed that 90% Zn + 10% Cd among the various mixtures of zinc and cadmium had highest toxicity with IC_{50} value of 0.098 ± 0.009 mM while 25% Zn + 75% Cd with IC_{50} of 0.514 ± 0.055 mM was least toxic. Their toxicity thresholds are significantly different. However, there is no significant difference in the toxic effect of 10% Zn + 90% Cd and Zinc as a single chemical. In the case of Zn + 4–CP binary mixtures, 50% Zn + 50% 4–CP concentration ratio showed highest toxicity which is not significantly different from toxicity of 75% Zn + 25% 4–CP mixture and their toxicity is comparable to toxicity of zinc alone. Other mixture ratios showed similar toxic effect with IC_{50} values not significantly different ($p>0.05$) from each other. Zinc and 2,4-DCP mixtures had comparable toxic effects with toxicity thresholds similar to effects of Zinc and 2,4-DCP as single chemicals except for mixture ratios of 90% Zn+10% 2,4-DCP and 50% Zn + 50% 2,4-DCP with highest and lowest toxic effects respectively.

The 90% Cd + 10% 4–CP mixture with IC_{50} of 0.113 ± 0.005 mM and 10% Cd + 90% 4-CP mixture with IC_{50} of 0.541 ± 0.04

mM had the highest and lowest toxicities respectively among the Cd + 4-CP concentration ratios. Binary mixtures of cadmium and 2,4-DCP showed relatively high toxicity with IC_{50} values that were lower than the values observed for the 2,4-DCP alone.

For the quaternary mixtures, highest and lowest toxicity were observed in 10% 2,4-DCP + 15% Cd + 30% Zinc + 45% 4-CP and 15% 2,4-DCP + 15% Cd + 45% Zinc + 25% 4–CP with IC_{50} values of 0.179 ± 0.006 mM and 0.252 ± 0.011 mM respectively.

The Toxic Index (TI): From the toxic index model analysis, synergistic, additive and antagonistic effects were observed in the mixtures as shown in Tables-3 and 4 respectively. The TI values ranged from 0.310 ± 0.014 to 2.050 ± 0.145, 0.781 ± 0.037 to 1.370 ± 0.026, 0.494 ± 0.022 to 1.259 ± 0.002, 0.437 ± 0.014 to 0.953 ± 0.009 and 0.407 ± 0.047 to 0.832 ± 0.054 for Zn + Cd, Zn + 4-CP, Zn + 2, 4-DCP, Cd+ 4-CP and Cd + 2,4-DCP binary mixtures respectively. The TI values for quaternary mixtures ranged from 0.464 ± 0.005 to 0.738 ± 0.000. The effect of 10% Zn + 90% Cd, 25% Zn + 75% Cd mixtures were antagonistic while the effect of 50% Zn + 50% Cd, 75% Zn + 25% Cd was synergistic. Additive effect was observed for 25% Zn + 75% 4-CP, 75% Zn + 25% 4-CP and 10% Cd + 90% 4-CP mixtures. Synergistic effect was observed for all the Cd + 4-CP and Cd + 2,4-DCP mixtures ratios. Zinc + 2,4-DCP mixtures showed antagonistic effect at all the mixtures evaluated except for 90% Zn + 10% 2,4-DCP that exhibited synergistic effect. All the quaternary mixtures evaluated showed synergistic effect according to the TI model.

Isobolographic analysis: The isobolographic analysis of the binary mixtures based on IC_{50} values of individual substances and their mixtures are shown in Figure-8. The isobolograms showed synergistic, additivity and in some cases antagonistic effects especially for most of the Zn + 2,4-DCP mixtures. The IC_{50} isoboles showed synergistic interaction for all Zn + Cd and Zn + 4-CP mixtures except for 10% Zn + 90% Cd, 25% Zn + 75% Cd and 90% Zn + 10% 4-CP mixture ratios. On the other hand, the isobologram of Zinc + 2,4-DCP mixtures indicated antagonistic interaction except for 90% Zn + 10% 2,4-DCP mixture. The isobologram of Cd + 4-CP and Cd + 2,4-DCP mixtures showed synergistic actions, with that of 10% Cd + 90% 4-CP lying within the additivity line.

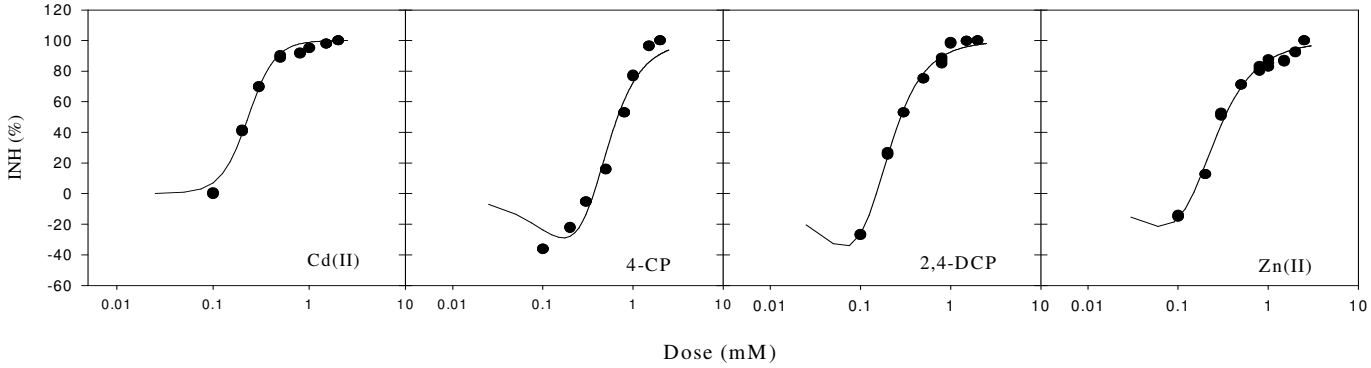

Figure-1: Toxicity of the single chemicals Cadmium, 4-CP, 2,4-DCP and Zinc to dehydrogenase enzyme activity of the bacterial consortium.

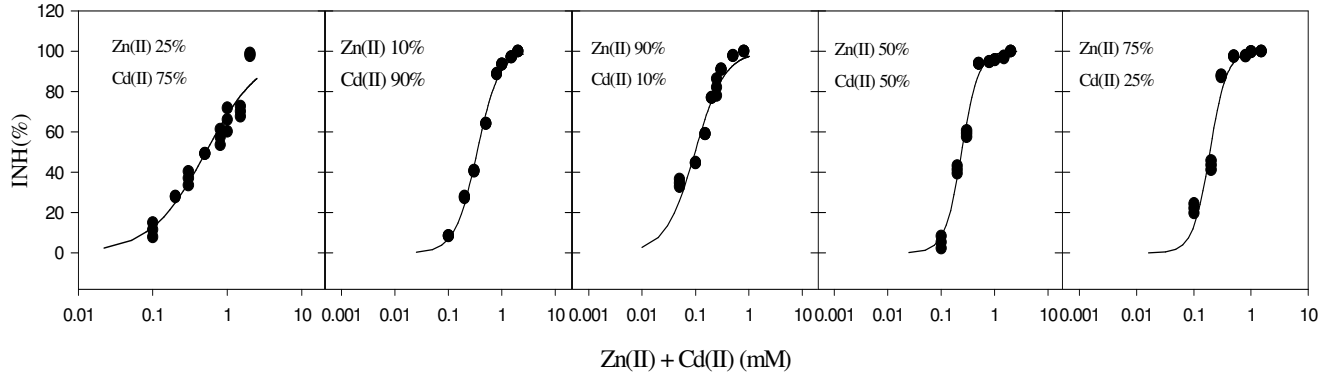

Figure-2: Toxicity of binary mixtures of Zinc (Zn) and Cadmium (Cd) to dehydrogenase enzyme activity of the bacterial consortium.

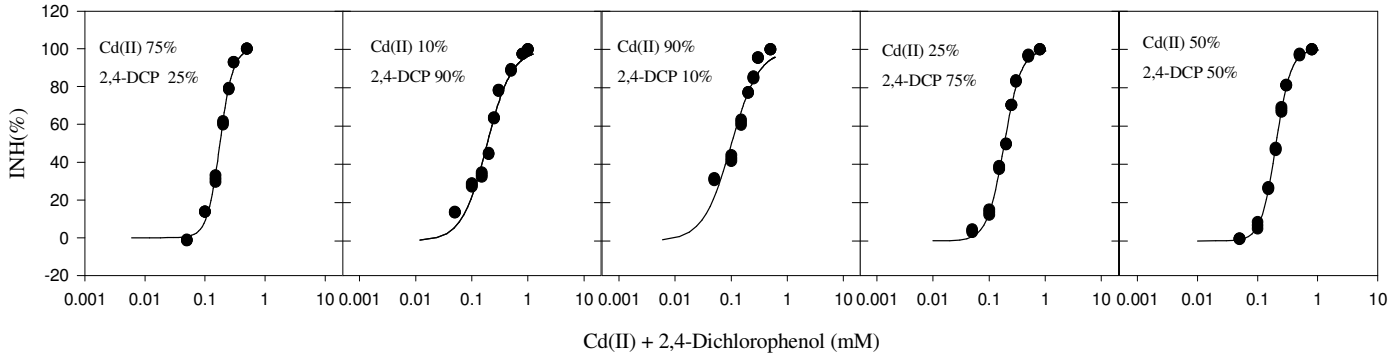

Figure-3: Toxicity of binary mixtures of Cadmium (Cd) and 2,4-DCP to dehydrogenase enzyme activity of the bacterial consortium.

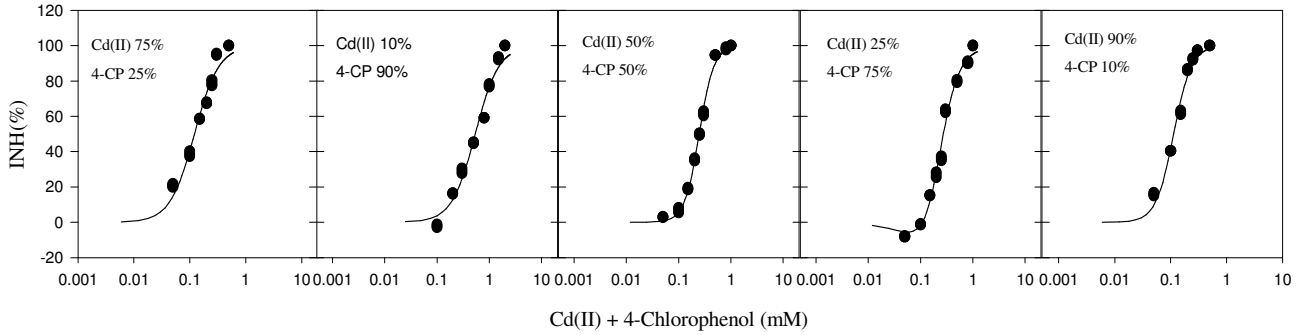

Figure-4: Toxicity of binary mixtures of Cadmium (Cd) and 4-CP to dehydrogenase enzyme activity of the bacterial consortium.

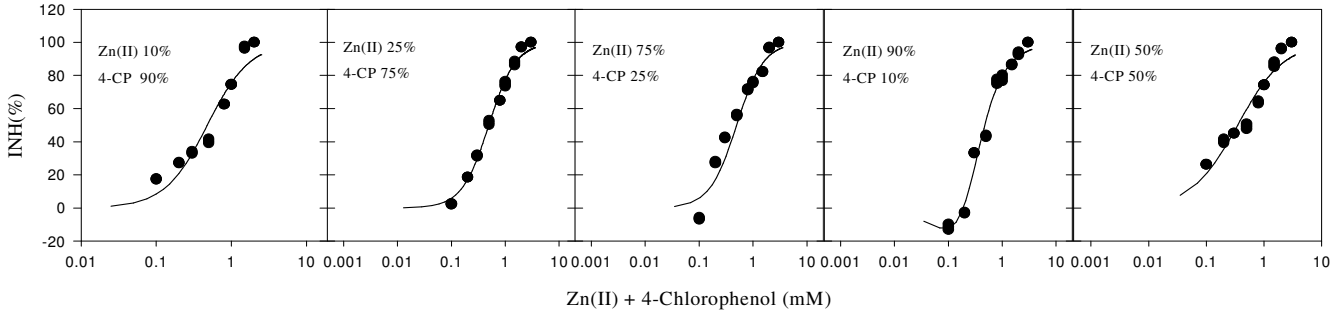

Figure-5: Toxicity of binary mixtures of Zinc (Zn) and 4-CP to dehydrogenase enzyme activity of the bacterial consortium.

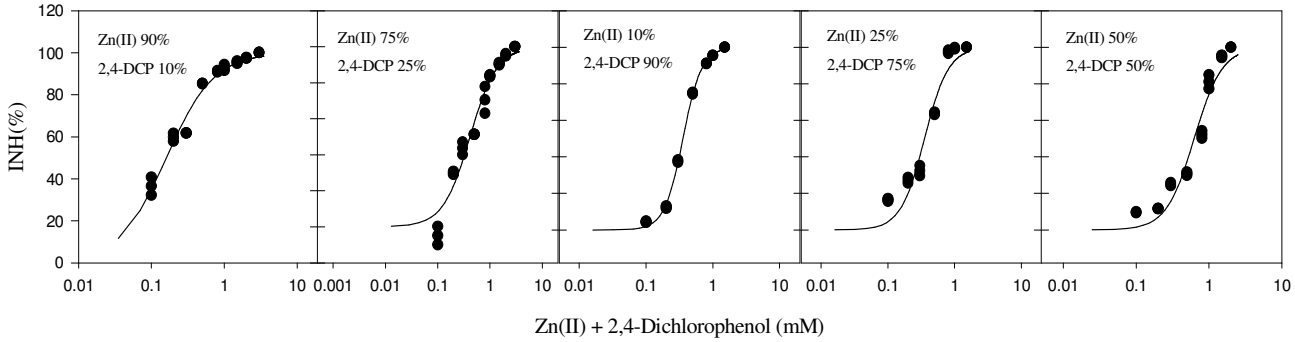

Figure-6: Toxicity of binary mixtures of Zinc (Zn) and 2,4-Chlorophenol (2,4-DCP) to dehydrogenase enzyme activity of the bacterial consortium.

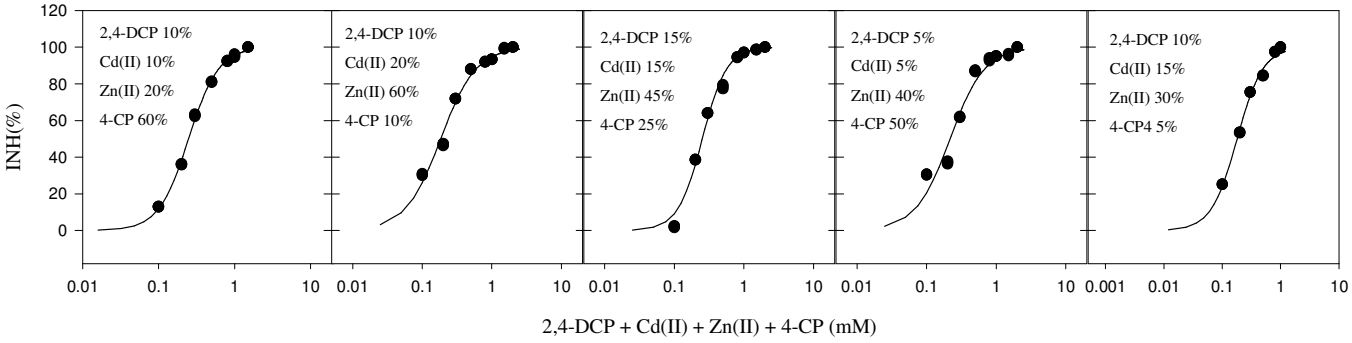

Figure-7: Toxicity of quaternary mixtures of the chemicals to the dehydrogenase activity of the bacterial consortium.

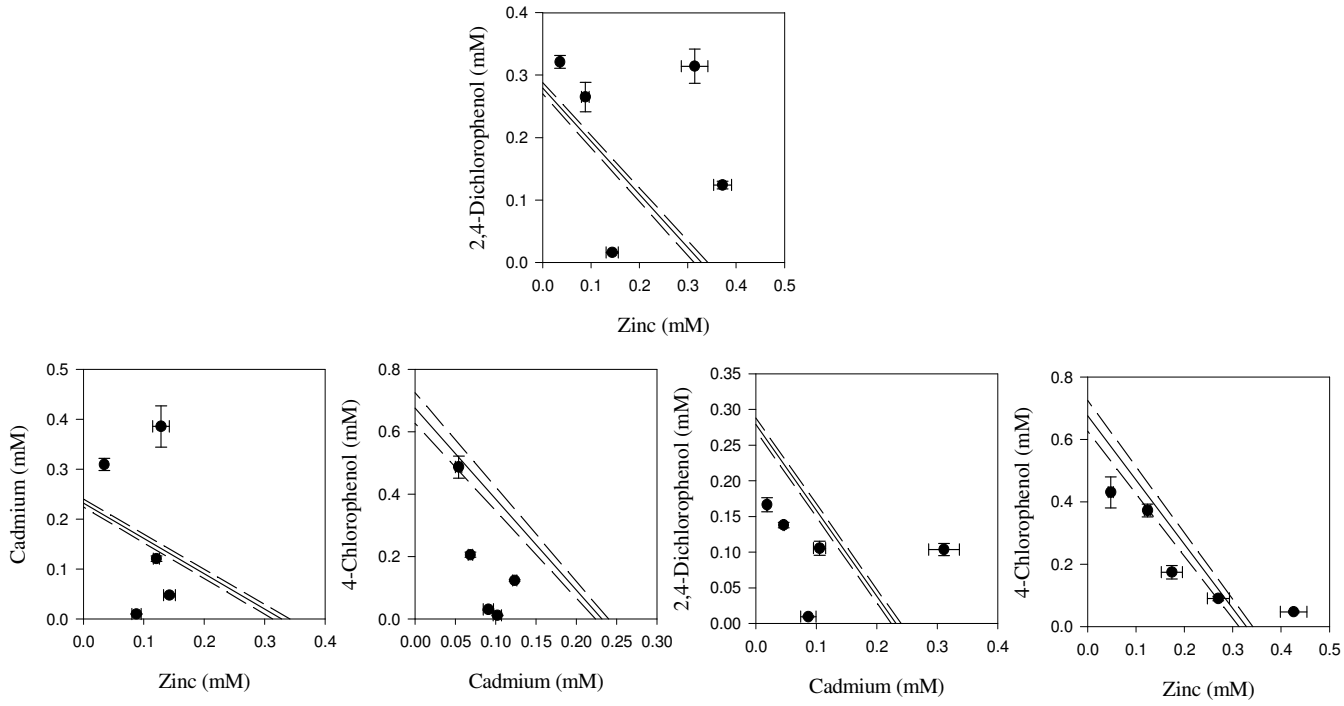

Figure-8: The IC_{50} isobologram for metals and phenols as individual and binary mixtures tested against dehydrogenase activity of bacterial consortium. The bars represent the standard deviations of the 95% confidence interval of the values. The solid and dotted lines represents additivity line and its 95% confidence belt.

Discussion: This study investigated the individual and combined action of the four toxic chemicals (Zn, Cd, 4-CP and 2,4-DCP) on dehydrogenase enzyme activity of bacterial consortium. In addition, Toxic Index model and isobolographic analyses were also applied on the data to ascertain the possible interactions of the toxic chemicals.

The dehydrogenase enzyme activity exhibited hormetic response upon exposure to heavy metals and phenolic compounds. Hormetic response to chemicals is a widely reported phenomena occurring in microorganisms and higher life[28]. The observed low dose stimulation (hormesis) in this study is in line with reported hormetic effect of metals and phenolic compounds. Nweke and co-workers reported low dose stimulation of dehydrogenase activity of *Rhizobium* species by glyphosate, 4-chlorophenol and 2,4-dichlorophenol[25]. Christofi reported hormetic response of immobilized bioluminescent *Vibrio fischeri* to low doses of phenol and 3,5-dichlorophenol[29]. Hormetic effect of 4-chlorophenol in this study is in line with report by Nweke and Okpokwasili[21], which showed low dose stimulatory effect at 20mg/l of 4-chlorophenol on dehydrogenase activity of *Pseudomonas* species. A time dependent hormetic effect of phenol on dehydrogenase activity was observed in *Bacillus* species, *Pseudomonas* species and

microbial community of petroleum refinery wastewater[12]. Stimulatory effect of 4-chlorophenol and 2,4-dichlorophenol on bacterial bioluminescence has been reported[24]. Stimulation at low doses of zinc in this study could be attributed to the role of zinc as essential element, participating as enzyme co-factor. Cadmium did not exhibit low dose stimulation. This is attributable to the fact that cadmium has no physiological function and is more toxic to microorganisms. Stimulatory role of zinc and phenol at low doses on yeast has been reported[30]. Environmental contaminations are frequently encountered as mixtures and behaviour of chemicals in a mixture may not correspond to the behaviors of individual chemicals[31]. The chemicals may interact and alter the toxicity of each other in a mixture. Modulation of toxicity of chemicals may have been established in this study with heavy metals and phenolic compounds evaluated.

Table-1: Toxicity threshold (IC_{50}) (mM) of the test chemicals in single and binary mixtures on dehydrogenase activity of bacterial consortium.

Test chemical and mixtures	IC_{50} (mM)
Zinc/Cadmium	
0:100	0.232 ± 0.008*c*
10%: 90%	0.344 ± 0.014*d*
25% : 75%	0.514 ± 0.055*e*
50% : 50%	0.242 ± 0.011*c*
75% : 25%	0.190 ± 0.013*b*
90% : 10%	0.098 ± 0.009*a*
100:0	0.328 ± 0.015*d*
Zinc/4-Chlorophenol	
0:100	0.675 ± 0.049*c*
10% : 90%	0.478 ± 0.055*b*
25% : 75%	0.496 ± 0.027*b*
50% : 50%	0.349 ± 0.044*a*
75% : 25%	0.360 ± 0.030*a*
90% : 10%	0.473 ± 0.030*b*
100:0	0.328 ± 0.015*a*
Zinc/2,4-Dichlorophenol	
0:100	0.279 ± 0.009*b*
10% : 90%	0.357 ± 0.012*c*
25%: 75%	0.353 ± 0.031*c*
50% : 50%	0.628 ± 0.054*d*
75% : 25%	0.359 ± 0.036*c*
90% : 10%	0.160 ± 0.014*a*
100:0	0.328 ± 0.015*c*
Cadmium/4-Chlorophenol	
0:100	0.675 ± 0.049*d*
10% : 90%	0.541 ± 0.040*c*
25% : 75%	0.274 ± 0.011*b*
50% : 50%	0.247 ± 0.005*b*
75% : 25%	0.121 ± 0.008*a*
90% : 10%	0.113 ± 0.005*a*
100:0	0.232 ± 0.008*b*
Cadmium/2,4-Dichlorophenol	
0:100	0.279 ± 0.009*e*
10% : 90%	0.185 ± 0.011*b*
25% : 75%	0.184 ± 0.005*b*
50% : 50%	0.201 ± 0.003*c*
75% : 25%	0.178 ± 0.004*b*
90% : 10%	0.099 ± 0.018*a*
100:0	0.232 ± 0.008*d*

Values with same letters are not significantly ($p > 0.05$) different.

Table-2: Toxicity threshold (IC_{50}) of the quaternary mixtures of the chemicals on dehydrogenase activity of bacterial consortium.

Mixtures (Ratio)	IC_{50}(mM)
10% 2,4–DCP : 10%Cd: 20%Zn : 60% 4–CP	0.248 ± 0.005*c*
5% 2,4–DCP : 5%Cd : 40%Zn : 50% 4–CP	0.217 ± 0.018*b*
10% 2,4–DCP : 15%Cd : 30%Zn : 45% 4–CP	0.179 ± 0.006*a*
15% 2,4–DCP : 15%Cd : 45%Zn : 25% 4–CP	0.252 ± 0.011*c*
10% 2,4–DCP : 20%Cd : 60%Zn : 10% 4–CP	0.184 ± 0.011*a*

Values with same letters are not significantly ($p > 0.05$) different.

Table-3: Toxic Index of the test chemicals in binary mixtures and their respective interactions on dehydrogenase activity of the bacterial consortium according to T1 model.

Mixtures	**TI**	**Effect**
Zinc/Cadmium		
10% Zn+ 90% Cd	1.438 ± 0.006	Antagonistic
25% Zn+ 75% Cd	2.050 ± 0.145	Antagonistic
50% Zn+ 50% Cd	0.889 ± 0.005	Marginally Synergistic
75% Zn+ 25% Cd	0.639 ± 0.017	Synergistic
90% Zn+ 10% Cd	0.310 ± 0.014	Synergistic
Zinc/4-Chlorophenol		
10% Zn+ 90% 4 CP	0.781 ± 0.037	Synergistic
25% Zn+ 75% 4-CP	0.930 ± 0.007	Additive
50% Zn+ 50% 4-CP	0.788 ± 0.056	Synergistic
75% Zn+ 25% 4-CP	0.957 ± 0.035	Additive
90% Zn+ 10% 4-CP	1.370 ± 0.026	Antagonistic
Zinc/2,4-Dichlorophenol		
10% Zn+ 90% 2,4-DCP	1.259 ± 0.002	Antagonistic
25% Zn+ 75% 2,4–DCP	1.218 ± 0.066	Antagonistic
50% Zn+ 50% 2,4–DCP	2.082 ± 0.012	Antagonistic
75% Zn+ 25% 2,4–DCP	1.141 ± 0.067	Antagonistic
90% Zn+ 10% 2,4–DCP	0.496 ± 0.022	Synergistic
Cadmium/4-Chlorophenol		
10% Cd+ 90% 4–CP	0.953 ± 0.009	Additive
25% Cd+ 75% 4–CP	0.600 ± 0.008	Synergistic
50% Cd+ 50% 4–CP	0.716 ± 0.017	Synergistic
75% Cd+ 25% 4–CP	0.437 ± 0.014	Synergistic
90% Cd+ 10% 4–CP	0.456 ± 0.006	Synergistic
Cadmium/2,4-Dichlorophenol		
10% Cd+ 90% 2,4 –DCP	0.676 ± 0.018	Synergistic
25% Cd+ 75% 2,4 –DCP	0.693 ± 0.004	Synergistic
50% Cd+ 50% 2,4 –DCP	0.832 ± 0.054	Marginally Synergistic
75% Cd+ 25% 2,4 –DCP	0.735 ± 0.008	Synergistic
90% Cd+ 10% 2,4 –DCP	0.407 ± 0.047	Synergistic

Table-4: Toxic interactions of quaternary mixtures of the chemicals on dehydrogenase activity of the bacterial consortium according to TI model.

Quaternary mixture ratios	T1	Interaction
10% 2,4–DCP + 10% Cd + 20% Zn+60% 4-CP	0.568 ± 0.018	Synergistic
5% 2,4–DCP + 5% Cd + 40% Zn+50% 4-CP	0.510 ± 0.015	Synergistic
10% 2,4–DCP + 15% Cd + 30% Zn+45% 4-CP	0.464 ± 0.005	Synergistic
15% 2,4–DCP + 15% Cd + 45% Zn+25% 4-CP	0.738 ± 0.003	Synergistic
10% 2,4–DCP + 20% Cd + 60% Zn+10% 4-CP	0.588 ± 0.009	Synergistic

The isobolographic analysis of the IC_{50} values as well as the TI model used to analyse mixture toxicity indicated similar results with regards to the toxicity of zinc, cadmium, 4-chlorophenol and 2,4-dichlorophenol mixtures against dehydrogenase activity of the bacterial consortium evaluated in this study. Although there were observed synergistic and antagonistic effect of the mixtures, the TI values between 0.639 ± 0.017 to 1.438 ± 0.006 for Zn + Cd mixtures, 0.781 ± 0.037 to 1.370 ± 0.026 for Zn + 4-CP mixtures, 1.218 ± 0.066 to1.370 ± 0.026 for Zn + 2,4-DCP mixtures, 0.600 ± 0.008 to 0.953 ± 0.009 and 0.676 ± 0.018 to 0.832 ± 0.054 for Cd + 4-CP and Cd + 2,4-DCP binary mixtures are within the range 0.5 to 2.0 proposed by Dener[32] as additive. Similar conclusions were made elsewhere[25,27]. Based on the TI analysis, the joint action of the mixture Zn + 2,4-DCP was antagonistic to the dehydrogenase activity of the bacterial consortium, which means that Zn interacted with the phenolic compound to reduce the toxicity of the mixture. In addition, the antagonistic effect was also observed for the mixtures of Zn + Cd. It has been suggested that Cd could induce oxidative damage to cells[33].

Antagonistic effect of 25%Zn + 75% Cd mixture observed in this study presumes that Zn(II) may reduce the toxicity of Cd(II) on dehydrogenase enzyme activity. Antagonistic interaction was observed by toxicity of Zinc and Cadmium binary mixtures on sea Urchin embryo while quaternary mixtures of Copper, Lead, Zinc and Cadmium were mainly addictive[34]. Synergistic interaction of binary combination of pentachlorophenol and copper was reported by Zhu and co-workers[35]. Similarly, quaternary mixtures of nonessential metals exhibited synergistic interaction[36]. Mowat and Bundy reported possibility of synergistic, additive and antagonistic interactions among binary and ternary mixtures of pollutants[37]. This is in agreement with the observations in this study. Toxicity interaction of zinc and cadmium on *Pseudomonas fluorescens* and *E. coli* was synergistic[22].

The environmental contaminants at increasing concentrations exert inhibitory effects on indigenous microorganisms of an ecosystem and thus disturb the ecosystem. The inhibition of dehydrogenase activity observed in this study at high concentrations of zinc, cadmium, 4-chlorophenol and 2,4-dichlorophenol, is consistent with what has been widely reported. Phenols are membrane damaging biocides[38-39] . They cause alteration in the integrity of cytoplasm and disrupts membrane functions. Since dehydrogenases are membrane associated, loss of membrane integrity will ultimately affect their functions[39]. In the present study, the order of toxicity, Cd > 2,4-DCP > Zn > 4-CP is in line with the report of Ren and Frymier[40]. Higher toxicity of 2,4-DCP when compared to 4-CP observed in this study corresponds to the findings of Rani et al. on degradation of mixture of phenolic compounds by activated sludge process using mixed consortia[41]. Increase in the number of substituents in the structure of phenol, increases its toxicity[42]. Less toxicity of pentachlorophenol and high toxicity of zinc and cadmium on increased pH on acetate and glucose mineralization has been documented[43]. The progressively decreasing stimulation of dehydrogenase activity with increase concentrations of 4-CP and 2,4-DCP observed in this study corresponds to the well reported toxic effect of phenols at elevated concentrations even for organisms that use phenols as energy source. Okolo and co-workers also reported progressive increase in inhibition of dehydrogenase activity and periplamic reductase enzymes of *Acinetobacter* species by phenolic compounds[39]. Higher toxicity of cadmium than zinc has been widely reported.

Conclusion

The aim of this study was to assess the toxicological impacts of zinc, cadmium 4- chlorophenol and 2,4-dichlorophenol in their singles, binary and quaternary mixtures on dehydrogenase activity of bacterial consortium. The pattern of toxicity of the chemicals after 24 h period can be ranked as Cadmium > 2,4-dichlorophenol > Zinc > 4-chlorophenol. Results of the study showed that high concentrations of the pollutants above hormetic range had profound effects upon the bacterial consortium dehydrogenase activity. The response of the enzyme activity to binary mixtures suggests that Cadmium and 2,4-dichlorophenol mixtures exhibited high toxicity. Toxic index and isobolographical analysis showed synergistic effect in most of the binary and all quaternary combinations evaluated. Low concentration of zinc and high concentration of cadmium mixtures lead to antagonistic effect while synergistic interactions occur vice versa. Cadmiun and 4-CP binary mixtures showed synergistic effect. However, the three effects (synergism, antagonism and additivity) occurred in Zinc and 4-CP mixtures.

Therefore the combined toxic effects of metals and phenols should be considered in the risk assessment of heavy metal and phenol pollution in both agricultural land and aquatic environment.

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