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Synergistic Toxicity of Phenols and Cadmium Ion Binary Mixtures on *Bacillus Sp* and *Pseudomonas Sp*

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Authors' contributions

There was total collaboration among all authors in the execution of this study. Authors CAMAF, JCO and CEN designed the study, author CAMAF, ESA and RCN performed the statistical analysis, authors JCO, CEN wrote the protocol and author CAMAF wrote the first draft of the manuscript. Authors CAMAF, JCO, CEN and ESA managed the analyses of the study. Author RCN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Toxicity of Cadmium (Cd), 4-chlorophenol (4-CP), and 2,4-dichlorophenol (2,4-DCP) as single compounds and binary mixtures on *Bacillus sp* and *Pseudomonas sp* isolated from garden soil was assessed using inhibition of total dehydrogenase as toxicity response. Binary mixtures of metal and chlorophenol were composed using arbitrary concentration ratios (%) corresponding to metal/phenol mixtures of 20/80; 40/60; 50/50 and 30/70; 45/55, 50/50 for the chemical pairs: Cd/4-CP and Cd/2,4-DCP respectively. Results obtained showed that the binary mixtures of Cd/4-CP and Cd/2,4-DCP all exhibited a dose-dependent inhibition of dehydrogenase activity in the test isolates. The binary mixture of CD/4-CP exhibited higher toxicity in ratio 40/60 and 20/80 for the isolates *Bacillus sp* and *Pseudomonas sp* with IC_{50} values of 0.212±0.002 and 0.158± 0.008mM

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respectively. While for the mixture of Cd/2,4-DCP, toxicity was highest at the 50/50 and 45/55 ratio for *Bacillus sp* and *Pseudomonas sp* with IC_{50} values of 0.069±0.001mM and 0.068±0.001mM respectively. The binary mixtures of the chemicals evaluated showed a progressive inhibition of dehydrogenase activity with *Pseudomonas sp* showing a higher susceptibility. Isobolographic analysis of binary mixture interaction against *Bacillus sp* and *Pseudomonas sp* showed largely synergistic interactions. The combination of Cd with the chlorophenols resulted in a synergistic increase in the toxicity of the compounds to the test isolates. The toxicity of Cd/4-CP binary mixture ratios to dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp* was in the order 40:60>50:50>20:80 and 20:80>50:50>40:60 respectively; similarly that of Cd/2,4-DCP mixtures were 50:50>45:55>30:70 and 45:55>50:50>30:70 for Bacillus and *Pseudomonas sp* respectively. The trend and implications of the toxicity interactions are further discussed.

Keywords: Toxieavcity; hy metals; phenols; synergism.

1. INTRODUCTION

Phenols are an essential part of the industrial production process and form the bulk residue in textile, petrochemical, and pharmaceutical industries effluent [1, 2]. They are mostly recalcitrant to microbial degradation and pose a significant risk to microbial diversity of terrestrial and aquatic environment as well as to man [3, 4]. Increased global population has led to a surging need for food; resulting in increased industrial and agricultural activities needed to meet these needs. The use of chemicals pesticides, fertilizers, and other inputs has led to the introduction of degradation products such as chlorophenol compounds; Chlorobenzenes, chlorinated cyclohexanes, and heavy metals into the environment [5-8]. The presence of chemicals in the environment due to their use for various purposes affects the quality of air, water, soil, and human health. Several studies have focused on assessing the attendant impact of human activities on other life forms in our ecosystem; to ensure that our drive to survive does not completely negate the existence of other life forms. They will help create a firm basis for environmental policy formulation. Environmental pollution and toxicant levels in effluents, industrial, domestic wastes, and agricultural wastes arising from human activities have been estimated by bioassays employing the micro and macro vertebrates [9,10]. Recent studies have focused on microbes as an alternative to test with animals [11]. The ubiquitous nature of microbial communities presents a sensitive and informative indicator of the presence of toxic substances in the environment. Microbes are prime mediators of biogeochemical cycling, adapted to exist on dissolved substances that are often present in the environment at very low concentrations [11]. Because of their versatility, some strains are capable of tolerating the presence of high concentrations of potentially harmful substances. Measurement of changes in microbial respiratory enzymes activities is a useful indicator for risk assessment [12]. Organo-chlorinated compounds and heavy metals are categorized as dangerous substances (based on their toxicity, stability, and bioaccumulation) that should be monitored in waters [13]. The environmental impacts of these compounds have been reported, but few studies have focused on metal-phenol co-contamination in the natural environment or microbial communities. Toxicity patterns and interaction of drugs and chemical mixtures have been widely reported and mathematically described in recent works [12, 14-21]. The present study seeks to assess the toxicity of cadmium and the phenolic derivatives, 4-chlorophenol and 2,4-dichlorophenol on the total dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp* isolates. *Pseudomonas sp* and *Bacillus sp* have been widely reported to be primary degraders of major environmental pollutants [22-25]. They immobilize or detoxify organic compounds, metals and radionuclides through various mechanisms [22-23]; involving complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds. This will not only determine toxicity but access how each compound act to modulate the toxicity of each other using a randomized arbitrary concentration ratio for their binary mixture composition.

2. MATERIALS AND METHODS

2.1 Reagents

The compounds used in this study include 4-Chlorophenol (Merck, Germany), 2,4-

dichlorophenol (JHD, China), Cadmium (Merck, Belgium), and 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, Germany). All other reagents used were of analytical grade.

2.2 Study Area and Collection of Sample

The microorganism used for the assay was isolated from uncultivated agricultural farmland with geographical location, 5^{o} $28^{1}40^{11}$N, $7^{o}2^{1}34^{11}$E located at Ihiagwa, Owerri West LGA of Imo State Nigeria. The farmland is characterized by household refuse dumps from students' hostels; subjected to sewage water run-off and other human and animal activities. The soil sample was collected randomly, about 4 inches depth from the soil surface according to Myers and Stokes [26] guidelines for uncultivated farmland. Using a clean, dry, sterile (70% alcohol) shovel, a four inches core was made and soil samples were collected using a sterile split-spoon to obtain a fresh soil sample within 5minutes and deposited into a sterile plastic container under room temperature within 24h.

2.3 Isolation of Bacteria Strains

About 5g of the soil sample was suspended in 100ml of sterile distilled water and homogenize for microbial isolation. A ten-fold serial dilution was carried out and 10^{-6} dilution cultured. About 0.1ml of diluted sample was plated out and spread on dry sterile nutrient agar and incubated for 24h. The isolation was carried out using the spread plate technique, and incubated at room temperature of (28 ± 2^{o}C) for 24hrs. The bacterial colonies that developed were purified by sub-culturing unto freshly prepared nutrient agar plates to obtain pure microbial colonies; which were subjected to biochemical test for identification.

2.4 Preparation of Inoculum for Toxicity Assay

The isolates for the study were prepared by growing in sterile nutrient broth on a rotary incubator (150rpm) at room temperature (28 ± 2^{0}C) for 24 hrs.

The cells were harvested by centrifugation and washing twice in sterile distilled water at 4000 rpm for 10 mins [27]. The pure packed cells were suspended in the sterile distilled water and standardized to optical density of 0.1 at 540 nm in a spectrophotometer (VIS 721D, Life Assisstance Scientific INST. CO).

2.5 Preparation of Mixtures / Design of Experiment

The binary mixtures of 4-Chlorophenol, 2,4-Dichlorophenol and Cadmium was composed using arbitrary concentration ratios (%) corresponding to metal/chlorophenol mixtures of 20/80; 40/60; 50/50 and 30/70; 45/55, 50/50 for the chemical pairs: Cd/4-CP and Cd/2,4-DCP respectively. The composite mixtures were created using 10mM stock of Cd, 4-CP and 2,4-DCP, and The mixtures were final stock concentration of mixture was 10mM.

2.6 Toxicity Assay

Determination of the inhibitory effect of single compounds and the binary mixtures on dehydrogenase activity of the isolates was according to the method described by Nweke et al., [27]. 2,3,5-triphenyltetrazolium chloride (TTC) was used as the artificial electron acceptor. The assay consisted of graded concentrations of the toxicants (i.e 4-Chlorophenol, 2,4-Dichlorophenol, Cadmium, binary mixtures of 20/80; 40/60; 50/50 and 30/70; 45/55, 50/50 for the chemical pairs: Cd/4-CP and Cd/2,4-DCP respectively; nutrient broth, isolates and TTC in 2ml volume. The requisite volume of respective toxicant stock was amended with sterile distilled water, a 0.5ml portion of 0.1% nutrient broth (x4 Strength) added to each 20ml screw capped test tube. Thereafter, 0.1 ml each of 0.1%w/v solution of TTC, and 0.1 ml bacterial suspension were added into each tube to obtain the final concentration. The final concentrations of the toxicants ranged from 0 to 2.5 mM. The controls consisted of the medium void of toxicants. The set-up was incubated at room temperature (28 ± 2^{0}C) for 24 hrs. Furthermore, 1ml of 1%v/v Triton X100 was added into each tube and allowed to stand for 10 min, the TTC-formazan produced in each tube was extracted in 4 ml of butanol; and absorbances of the extracts were determined spectrophotometrically at 500nm.

2.7 Data Analysis

The inhibition of total dehydrogenase activity at varying concentrations of the 4-CP, 2,4-DCP, and mixtures were calculated as shown in equation (1). The responses were calculated

from triplicate determinations as described by Nweke et al., [27].

$$\%INH = \frac{C_A - T_A}{C_A} \times 100 \quad \ldots\ldots(1)$$

Where C_A represents the absorbance of TPF extract in the control; T_A is the absorbance of TPF extract in the test with different concentrations of the single and binary mixtures.

2.8 Determination of Toxicity Threshold

The dose-response data generated was further fitted into 4-parameter logistic dose-response model (LDR (a,b,c,d) (equation 2), to obtain their respective ecotoxic threshold (EC_{50}) which is defined as the concentrations of the toxicants that inhibited the dehydrogenase activity of the isolate by 50%.

$$y = a + \frac{b}{1+\left(\frac{x}{c}\right)^d} \quad (2)$$

Where x represents the concentration of the fraction, a and b are the minimum and maximum response of negative control respectively, c represents the IC_{50}, d is a constant that determines the slope at IC_{50}.

2.9 Isobolographic Analysis of the Mixture Toxicities

The isoboles of the binary mixtures were used to determine the combined effects of the mixtures as stated in Nweke et al. [27]. TU_i is the toxic unit of each of components of the mixtures. The toxic unit of each component is computed using the expression:

$$TU_i = \frac{Cmix_i}{IC_{50i}} \quad \ldots\ldots(3)$$

Where $Cmix_i$ is the concentration of the *i*th toxicant in the mixture and IC_{50i} is the IC_{50} of the same toxicant when tested alone.

3. RESULTS AND DISCUSSION

3.1 Identification of Bacterial Isolates

The results of the morphological and biochemical characteristics of the microorganisms isolated from the soil sample the organisms were tentatively identified as *Pseudomonas sp* and *Bacillus sp*.

3.2 Toxicity of Cadmium, 4-Chlorophenol, and 2,4-dichlorophenol on the dehydrogenase Activity of *Bacillus sp* and *Pseudomonas sp.*

Fig. 1 shows the logistic dose response curve of toxicity of Cadmium, 4-Chlorophenol, and 2,4-dichlorophenol on the dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp.* The results showed a progressive inhibition of dehydrogenase activity with increasing concentration of the toxicants (Cd, 4-CP and 2,4-DCP) against the two isolates. However, Stimulation of dehydrogenase activity of *Bacillus sp* was observed at low doses of 0.1 – 0.25mM for 2,4- DCP and <0.1mM 4-CP. The toxicity of the compounds showed saturation effect at 0.8mM, beyond which the net toxicity did not considerably vary as inhibition of *Bacillus sp* and *Pseudomonas sp.* dehydrogenase activity approached 100%.

3.3 Toxicity of Binary Mixtures of Cadmium/4-CP and Cadmium/2, 4-DCP on *Bacillus sp* and *Pseudomonas sp.*

Fig. 2. shows the results of the toxicity of binary mixtures of Cadmium/4-CP and Cadmium/2, 4-DCP on *Bacillus sp* and *Pseudomonas sp*. The binary mixtures of Cd/4-CP in the ratios 20%:80%; 40%: 60%, 50%:50% and Cd/2,4-DCP in the ratios 30/70; 45/55, 50/50 all exhibited a similar toxicity trend, showing a concentration dependent inhibition of dehydrogenase activity in the isolates. The metal/phenol binary mixtures caused a shift in toxicity, with saturation occurring at 0.5mM and 0.2mM in the Cd/4-CP and Cd/2,4-DCP mixtures respectively, beyond which the net toxicity did not considerably vary as toxicity of the mixtures on *Bacillus sp* and *Pseudomonas sp.* approached 100%.

3.4 Median Inhibitory Concentrations (IC_{50})

The median inhibitory concentrations (IC_{50}) of the single and binary mixtures are presented on Table 1. The results showed that Cadmium had the highest toxicity with an IC_{50} value of 0.152±0.001mM, while 4-Chlorophenol showed

the least toxic effect with IC_{50} value of 0.525±0.002 mM against *Bacillus sp*; however, similar trend was also seen against *Pseudomonas sp.* with IC_{50} values of 0.139±0.001mM and 0.251±0.002mM for Cadmium and 4-CP respectively. The toxicity of the chemicals determined singly were in the order Cd^{2+}>2,4-Dichlorophenol>4Chlorophenol. Furthermore, Cadmium/ 2,4-DCP mixture showed a higher toxicity for all the mixture ratios studied than Cadmium/4-CP mixtures for both isolates.

3.5 Isobolographic Analysis

The isobolographic analysis of the mixtures using IC_{50} values of single toxicants and the binary mixtures against the two isolates are presented in Fig. 3.0. The isobolograms indicated chiefly synergistic interaction of the metal/chlorophenol components in the different binary mixture ratios. However, in the Cd/4-CP 50%:50% mixture, the interaction was marginally synergistic in both isolates, with the TU value closely approaching the additivity line.

The present study investigated the toxicity of binary mixtures of Cadmium and phenols to *Pseudomonas sp* and *Bacillus sp* isolated from garden soil. The toxicity of heavy metals to the environmental microbial community has been widely reported [28.-30]. *Bacillus sp* has been reported as a susceptible marker of heavy metal toxicity under oligotrophic conditions [31]. In the present study, the dose-response relationship of cadmium ions (Cd^{2+}) and the chlorophenolic compounds (2,4-DCP and 4-CP) on dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp* studied were different in various mixtures compositions. The result of 2,4-DCP toxicity on *Pseudomonas sp* and *Bacillus sp* showed a hormetic response curve which was characterized by stimulation of enzyme activity at low concentration (< 0.1mM); above the stimulatory range, the phenolic compound progressively inhibited dehydrogenase activity.

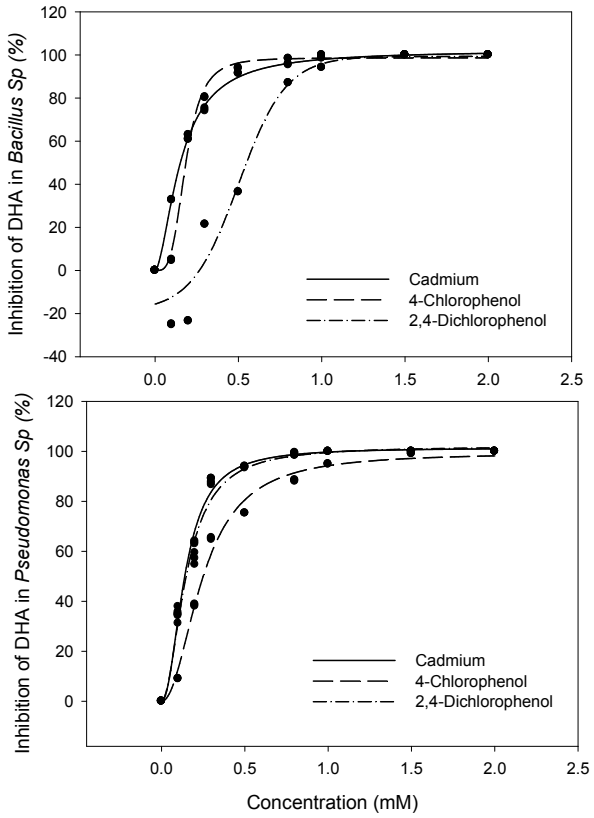

Fig. 1. Toxicity of Cadmium, 4-Chlorophenol and 2,4-dichlorophenol on dehydrogenase activity of *Bacillus sp.* and *Pseudomonas sp.*

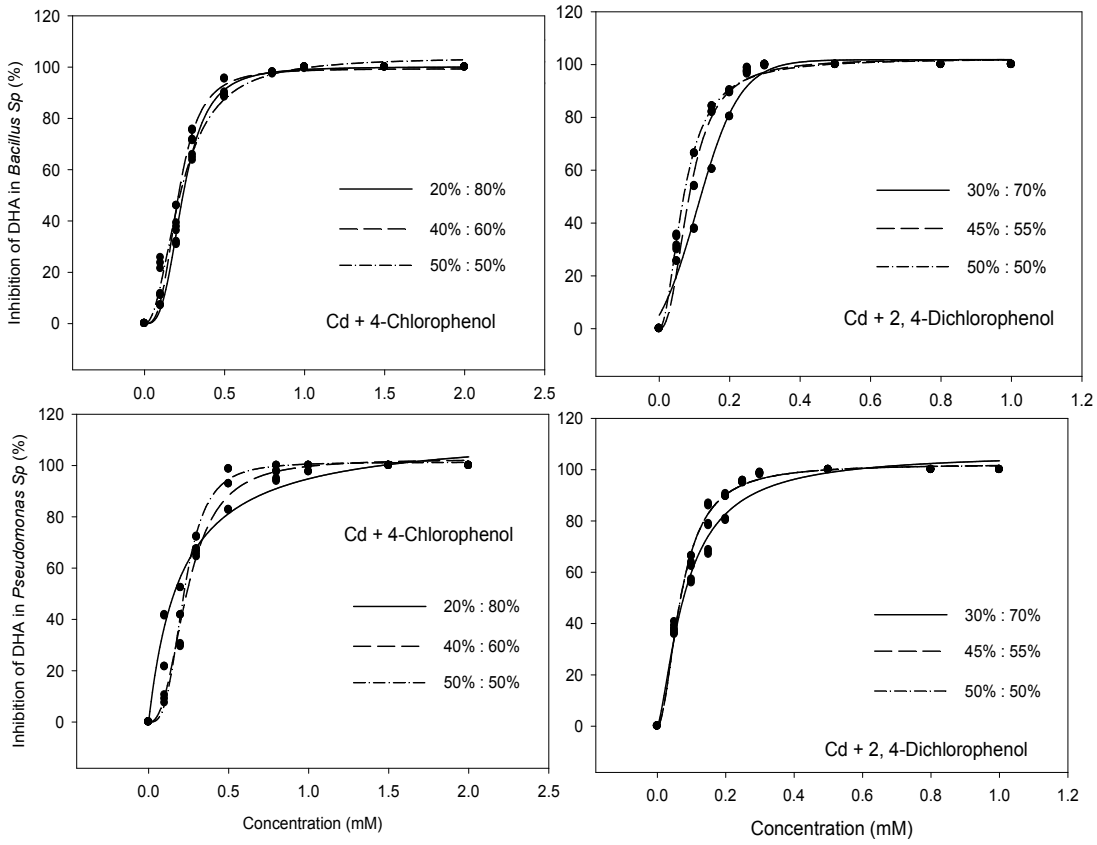

Fig. 2. Toxicity of arbitrary concentration ratio binary mixtures of Cadmium and 4-Chlorophenol and Cadmium and 2,4-dichlorophenol to *Bacillus sp.* and *Pseudomonas sp.*

Table 1. Median inhibitory concentrations (IC_{50}) of toxicant mixtures

		Median Inhibitory concentration (IC_{50})				
Bacteria Isolates	Mixtures	100%: 0%	20% : 80%	40%: 60%	50%:50%	0% : 100%
Bacillus sp.	Cd/4CP	0.152±0.001	0.240±0.001	0.212±0.002	0.221±0.002	0.525±0.002
Pseudomonas sp.		0.139±0.001	0.158±0.008	0.238±0.005	0.220±0.002	0.251±0.002
		100% : 0%	30% : 70%	45% : 55%	50% : 50%	0% : 100%
Bacillus sp.	Cd/ 2,4-DCP	0.152±0.001	0.106±0.001	0.085±0.001	0.069±0.001	0.176±0.001
Pseudomonas sp.		0.139±0.001	0.077±0.001	0.068±0.001	0.069±0.001	0.148±0.002

Thus, the stimulatory effect of this compound at a low dose suggests tolerance of 2,4-DCP by the microorganism. A time-dependent hormetic effect of phenol on dehydrogenase activity of *Bacillus sp*, *Pseudomonas sp*, and microbial community of petroleum refinery waste-water has been reported [32]. The study also showed the order of increasing toxicity of the individual chemical to dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp* was Cd^{2+}> 2,4-DCP>4-CP. The higher toxicity of 2,4-DCP when compared to 4-CP observed in this study agrees with the findings of Nwanyanwu *et al.,* [33], on inhibition of dehydrogenase activity of *Pseudomonas sp* and *Bacillus sp*. Biphasic toxicity of phenolic compounds are characteristic of phenol utilizing bacteria [34-36]. Okolo *et al.,* [37] also reported a progressive increase in inhibition of dehydrogenase activity and periplasmic reductase enzymes of *Acinetobacter sp* by phenolic compounds. 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. Researches

on Cadmium revealed toxicity to bacteria, algae, fungi, plants, and humans [38-40].

In the mixtures, the joint effects of the compounds in binary mixtures showed that Cadmium in a mixture with the phenolic compound resulted in a higher inhibitory effect with an increase in the concentration of Cadmium as observed by Nwanyanwu *et al.,* [33].

The results of the toxicity of Cadmium and 4-CP to dehydrogenase activity of *Bacillus sp* and *Pseudomonas sp* in ascending order of mixture ratio was 40:60>50:50>20:80 and 20:80>50:50>40:60 respectively. Also, the toxicity of Cadmium and 2,4-DCP mixtures showed toxicity of mixture ratios to be 50:50>45:55>30:70 and 45:55>50:50>30:70 to *Bacillus sp*. and *Pseudomonas sp* respectively.

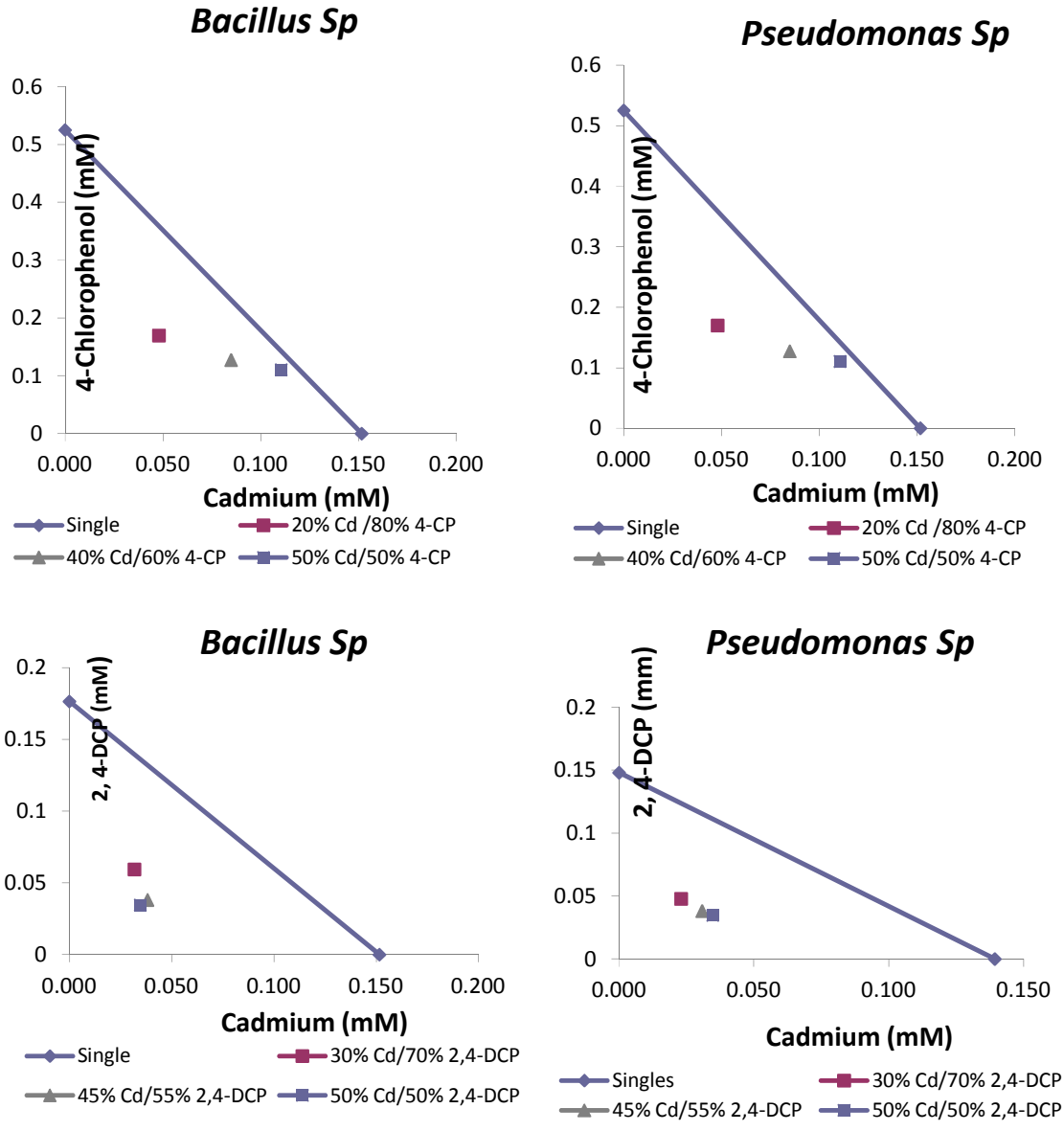

Fig. 3. Toxic Unit Isoboles of Cadmium/4-CP and Cadmium/2, 4-DCP on *Bacillus sp* and *Pseudomonas sp*

The IC_{50} Isobologram for metal and phenolic compounds in binary mixtures showed synergistic interactions for all the chemical mixtures. The line as shown in Fig. (3) represents additivity and the plots represented the different mixture ratios. However, the different responses of the bacterial isolates to the toxicity of these chemicals may be attributed to their source or isolation points. Thus, they have been proven to be reliable in the assessment of chemical toxicities [27]. The observed increase in toxicities of chemicals at high concentrations when applied singly is consistent with many reported cases of microbial response to single chemical exposure [12,17,41-42].

The dehydrogenase enzyme activity exhibited a hormetic response upon exposure to 2,4-DCP compounds. Hormetic response to chemicals is a widely reported phenomenon occurring in microorganisms and higher life [43]. The observed low dose stimulation (hormesis) in this study is in line with the reported hormetic effect of phenolic compounds. Nweke et al. [27] reported low dose stimulation of dehydrogenase activity of *Rhizobium sp* by 2,4-dichlorophenol. Environmental contaminations are frequently encountered as mixtures and the behavior of chemicals in a mixture may not correspond to the behaviors of individual chemicals [44]. The chemicals may interact and alter the toxicity of each other in a mixture.

4. CONCLUSION

The present study has demonstrated the toxicity of cadmium, 4-Chlorophenol, 2, 4-dichlorophenol binary mixtures against *Pseudomonas sp*. and *Bacillus sp*. The result showed a characteristic low dose stimulation of growth by 2,4-dichlorophenol. Binary mixtures of Cadmium with 4-Chlorophenol, and 2,4-Dichlorophenol showed a significant synergistic interaction in all the mixture ratios studied. Therefore, the presence of this mixture of compounds in effluent discharge into the environment poses a considerable risk to microbial diversity.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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