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Analysis of metal mixture toxicity to *Leptothrix* sp. using fixed ratio ray experimental design

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Abstract
There has been associate degree increasing ecological and world public health concern related to environmental contamination by metals in recent years. The current study is aimed toward analyzing metal mixture toxicity to *Leptothrix* sp. The various quantitative relation of toxicities of binary [Pb (II) + Ni (II), Cd (II) + Ni (II)] and ternary [Cd (II) + metal (II) + Ni (II)] mixtures of metals in addition because the toxicity impact of individual metal ions to *Leptothrix* sp. were assessed by using inhibition of dehydrogenase activity as associate degree finish purpose. Uniform design concentration relation (UDCR) and equieffect concentration relation (EECR) mixtures were designed to evaluate the combined toxicities of those significant metal ions. All the dose-response relationships of the UDCR and EECR mixtures and also the individual metal ions were delineating by logistical operates. The EC_{50} of the individual metal ion and their mixtures were calculable using logistical function. The Duncan test take a look at indicated considerably totally different EC_{50} values for every of the metal ions. Isobolographic and toxic index analyses showed doable interaction among the metal mixtures. The metal ions progressively inhibited the dehydrogenase activity because the concentrations were increased, giving percent inhibitions bigger than 95 % at 0.2 mM Cd(II) and 0.4 mM Pb (II). The percentage inhibition of Ni (II) was greater than 85% at 0.06 mM. The binary and ternary mixture ratio shows inhibitory effect of the dehydrogenase enzyme activity as the concentration incresases. Generally, the binary mixtures Cd (II) + Ni (II) mixture were antagonistic, Pb (II) + Ni (II) were Synergistic expect for percentage mixture of Pb (II) 31.21% + Ni (II) 68.79% that is additive. The Cd (II) 20 % + Ni (II) 80 % and Pb (II) + Ni(II) EECR 50 were additive. The ternary mixtures were all antagonistic. It is clear that heavy metals are the major sources of environmental pollution sometimes caused by anthropogenic activities; thus it is necessary for industries to treat these effluents before its discharge to the environment.

Keywords
Heavy metals, dehydrogenase activities, toxicity concentration addition model, independent action model.

Introduction

Environmental pollution is the presence of a pollutant (possibly heavy metal) in the environment (air, water and soil) that can be toxic and will be harmful to organisms living in the contaminated environment pollution (Chellaiah, 2018). Wastewater from industries, refineries and waste treatment plants is a direct cause of heavy metal pollution; heavy metal pollution occurs indirectly through pollutants entering the source; water supply from soil/groundwater systems and the

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atmosphere by rainwater (Vijayaraghavan and Yun, 2008). Heavy metals are common and persistent environmental pollutants released into the environment by industrial activities. Heavy metals pollute natural habitats and alter macro and microbiological communities (Nwekw et al., 2006; Horsfall and Spiff, 2002). Although heavy metals are naturally occurring elements found throughout the earth's crust, most environmental pollution and human exposure are as the result of human activities such as mining and smelting operations, industrial production, domestic and agricultural use of metals and metal-containing compounds (Begum and Huq, 2016). The major health concern worldwide is the indiscriminate release of heavy metals into the soil and waters; they cannot be broken down into non-toxic forms and therefore have long-term effects on the ecosystem. Many of them are toxic even at very low concentrations (Nlemolisa et al., 2020). Heavy metals can be grouped into essential metals such as copper, manganese, zinc and iron, and non-essential metals such as cadmium, lead, mercury and nickel (Graz et al., 2011). Cadmium and lead are among the main pollutants due to their high toxicity (Blandez et al., 2000; Carrillo-Gonzalez and Gonzalez-Chavez, 2012; Jaeckl et al., 2005). Soil, which is heterogeneous in nature, contains many microhabitats that are suitable for microbial growth. Soil microorganisms play an important role in maintaining the balance of soil biogeochemical cycles and soil fertility. They are also involved in various environmentally beneficial applications including bioremediation, biodegradation, biofuel production and bioleaching of mining ore tailings and wastewater treatment (Muniswamy et al., 2016). Human-induced activities, such as rural development and industry, agricultural pesticides, and ore residues release toxic heavy metals into the soil (Govil et al., 2008). The increase in mining activities has augmented the incidence of percolation of toxic metal ions infiltration into the surrounding ecosystem to alarming levels (Kulshrestha et al., 2013). The random dumping of hazardous waste in industrial zones can be a major cause of groundwater and soil pollution (Bhagure and Mirgane, 2011). Toxic metal pollution has the potential to affect soil microbial diversity by inhibiting physiological mechanisms such as respiration, Mg^{2+} transport, sodium-potassium ions and also reducing growth Vijayalakshmi et al., 2011).

In biological systems, heavy metals have been reported to affect cell organelles and components such as cell, membranes, mitochondria, lysosomes, endoplasmic reticulum, nucleus, and several enzymes involved in metabolism, detoxification, and damage repair (Wang et Shi, 2001). Metal ions have been shown to interact with cellular components such as DNA and nuclear proteins, causing DNA damage and structural changes that can lead to cell cycle modulation, carcinogenesis, or apoptosis (Chang et al., 1996). Heavy metal toxicity involves several mechanisms including; disrupts the enzymatic fuctions, acts as redox catalysts in the production of reactive oxygen species (ROS), disrupts ionic regulation and directly affects the formation of DNA as well as proteins (Gauthier et al., 2014; Hildebrandt et al., 2007). Due to the adverse effects of heavy metals, there is growing concern regarding the monitoring and research of metal pollution in ecological environments (Balogh and Salanki, 1987). Therefore, there is a need to develop sensitive, efficient and inexpensive methods for monitoring heavy metal concentrations in the environment. Heavy metal pollution in the environment is often characterized by mixtures of different metals, and therefore microorganisms living in contaminated sites are often exposed to mixtures of toxic metals in varying concentrations (Weltje, 1988). There is concern that exposure of organisms to chemical mixtures may lead to undesirable effects at low concentrations (Baas et al., 2007), and in recent years there has been a tendency to seek general behavior of chemical mixtures (Baas et al., 2007). In this study, we analyzed the toxicity of the metal mixture to *Leptothrix* sp. using fixed ratio ray experimental design.

Materials and methods

Sample collection

Soil samples for test organism isolation were aseptically obtained from a farm yard at Irette in the Owerri West Local Government Area, Imo State, Nigeria. Soil samples were taken with a sterile cylindrical instrument (auger) from a depth of 15-30 cm below the soil surface. Samples are transferred into sterile polyethylene bags and immediately transported to the laboratory for analysis.

Sample preparation and isolation

Ten fold serial dilution of the soil sample was prepared by weighing out 10 g of the soil sample and suspending it in 100 ml of sterile physiological saline to form the stock. The mixture was stirred for 1 min to allow the microbial cells to separate from the soil particles. Using

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a sterile pipette, one milliliter (1.0 ml) of the stock was pipetted and transferred into a sterile test tube containing 9 ml of sterile physiological saline to obtain 10^{-1}dilution. From this, it was subsequently diluted up to the 10^{-8} dilution. Then, 0.1 ml of 10^{-5}, 10^{-6} and 10^{-7} dilutions was plated in triplicate on MES-buffered minimal medium (Nlemolisa wt al., 2020). These plates were then incubated at 30 °C for 48 h.

Screening to select the best performing organism. After incubation, the best performing organisms capable of degrading INT were selected by adding one drop of 0.1% Iodo Nitro tetrazolium chloride (INT). Organisms that degraded INT by turning red were selected.

Purification of selected organisms. The distinctive colonies that degraded INT were picked.They were purified by repeatedly sub culturing and incubating at 30 °C for 48 h on fresh MES-buffered minimal agar. Purified isolates were subcultured in a MES-buffered minimal agar slant and stored in a 4°C refrigerator for further identification and testing.

Identification of Selected Organisms. Selected microorganisms were identified based on morphological, microscopic and biochemical characteristics (assimilation and fermentation) according to Bergey's Manual of Systemic Microbiology (Staley, 1989).

Preparation of inoculum. The bacterium was grown to mid exponential phase in nutrient broth (Lab M) on a rotary incubator (150 rpm) at room temperature (28 ± 2 °C) and cells were harvested by centrifugation at 3000 rpm for 10 min. Harvested cells were washed twice in sterile deionized distilled water to avoid nutrient transfer. The washed cells were re-suspended in sterile deionized distilled water and adjusted the optical density to 0.1 at 540 nm using a spectrophotometer. The cell suspensions were used as inoculum in toxicity assay.

Preparation of stock solutions of reagents. A 10 mM stock solution of the test chemicals (cadmium, nickel and lead) were prepared by dissolving 256.51 g/mol, 237.69 g/mol and 331.21 g/mol of each metal granules respectively in 50 ml of sterile deionized water and the volumes were brought to 100 ml in 100 ml volumetric flasks. The metal mixture was prepared by mixing and diluting the stock solution to obtain the desired combination of metal concentrations (0.1 mM and 1.0 mM) in the mixture. The stock concentrations of each metal of 10 mM, 1.0 mM and 0.1 mM were used to assay the single and metals mixtures.

Toxicity mixture assay

Fixed rays ratio design. Toxicity of binary mixtures of Cd (II) and Ni (II), Pb (II) and Ni (II) were determined using a fixed-ratio design, uniform design-concentration ratio (UDCR) and equivalent-effect concentration ratio (EECR). While keeping the mixing ratio constant, the total concentration of the mixture is varied to obtain a complete dose-response relationship of the mixture. The concentration ranges of the mixtures are based on the concentration ranges of the individual metals that give percentage inhibition ranging from 0% to 100% or close to 100%. Ni (II), Cd (II) and Pb (II) concentrations ranged from 0.1 to 10 mM as shown in Table 1 below.

Mixtures	Specific Ratio (%)					
Binary	EE_{20}		EE_{50}		EE_{80}	
Cd + Ni	Cd	Ni	Cd	Ni	Cd	Ni
	53.85	46.15	57.01	42.99	60.10	39.90
Pb + Ni	Pb	Ni	Pb	Ni	Pb	Ni
	72.73	27.27	31.21	68.79	33.45	66.55

Table 1. T*he specific ratios of metal ions in the binary mixtures (EECR20. EECR50 and EECR80)*

Binary mixtures of Cd (II) and Ni (II) were studied at the following weight-to-weight ratios: p(%) = 20, 50 and 80 of the Cd (II) concentration series, and 100-p (%) of the Ni (II) concentration series corresponding to the ratio of Pb (II): Ni (II) ration of : 20:80%, 50:50% and 80:20 %. were determined. over their concentration ranges. Binary mixtures of Pb(II) and Ni(II) were studied at the following weight-to-weight ratio: p(%) = 20, 50 and 80 of the Pb(II) concentration range, and 100 - p (%) of the range of Ni(II) concentrations corresponding to Pb(II): Ni(II) ratios of: 20:80 %, 50:50 % and 80:20 % based on their concentration series. Three different mixing ratios are considered based on the relative toxicity of the individual metals. First, a mixture in which the components was mixed in the ratio of the EC_{20} of the individual metals (known as EECR-20 mixture). Second, the toxicity of the mixture based on EC_{50} (EECR-50 mixture). Third, the toxicity of the mixtures based on EC_{80} (EECR-80 mixture) of

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individual metals was evaluated. The EC_{20}, EC_{50} and EC_{80} equieffect mixtures of Cd(II) and Ni(II) gives an effective ratios of 53.85 % Cd(II):46.15 % Ni(II); 57.01 % Cd(II):42.99 % Ni(II) and 60.10 % Cd(II):39.90 % Ni(II) respectively. The EECR-20, EECR-50 and EECR-80 equitoxic mixtures of Pb(II) and Ni(II) gave an effective ratios of 72.73 % Pb(II):27.27 % Ni(II), 31.21% Pb(II): 68.79% Ni(II) and 33.45% Pb(II): 66.55% Ni(II) respectively. Using the concentration ranges described for the binary mixture, the tertiary mixture of Cd(II) + Pb(II) + Ni(II), were also designed for a fixed ratio rays. Three homogenous design concentration ratios (UDCR) and three equieffect concentration ratios (EECR-20, EECR-50 and EECR-80) were evaluated. The specific ratios of metal ions in binary and tertiary mixtures are shown in Table 2.

Mixtures	Specific Ratio (%) Uniform Design Concentration Ratio								
Ternary	A			B			C		
Cd + Pb +Ni	Cd	Pb	Ni	Cd	Pb	Ni	Cd	Pb	Ni
	30	40	30	30	50	20	50	10	40
	Equivalent Effect Concentration Ratio								
Cd + Pb +Ni	EE_{20}			EE_{50}			EE_{80}		
	Cd	Pb	Ni	Cd	Pb	Ni	Cd	Pb	Ni
	24.14	55.17	20.69	30.5	46.5	23.0	36.2	39.7	24.1

Table 2
The specific ratios of metal ions in the ternary mixtures (UDCR, EECR20. EECR50 and EECR80)

Toxicity assay. Dehydrogenase activity was measured using a colorimetric test with iodo nitro tetrazolium chloride (INT) as the artificial electron acceptor. Inhibition of dehydrogenase activity was determined using iodo nitro tetrazolium chloride (INT) as an artificial electron acceptor that is reduced to red-coloured triphenylformazan (TPF). The reaction mixture consisted of a final volume of 2 ml of nutrient broth (NB) medium and INT supplemented with different concentrations of metal ions (pH 7.2) for individual metals and metal mixtures. Into each of the tubes, 0.5 ml of X4 strength (0.2% w/v) nutrient broth, 0.1 ml of INT (0.1% w/v), 0.1 ml of standardized bacterial suspension, required amount of distilled water, and stocks solution of each metal ion(s) were added to each 20 ml screw capped glass test tube to obtain various concentrations of metal ions for each metal and mixture ratios. Each concentration of mixtures and individual metals were prepared in triplicate. Controls were prepared without the toxicants. The cultures were incubated at room temperature (28±2° C.) for 48 h. After incubation, 1 ml of 1% v/v Triton X-100 was added to each tube, shaken for 1 min, and left at room temperature for 10 min. The red coloured INT-formazan (TPF) produced in each tube was then extracted with 4 ml of butanol. The absorbance of the extract was measured with a spectrophotometer at 500 nm.

Estimation of response. Inhibition of dehydrogenase activity at different concentrations of the individual, binary and ternary mixtures of nickel, cadmium, and lead were calculated relative to controls using equation [1].

$$\%IHN = \frac{C_A - T_A}{C_A} \times 100 \qquad [1]$$

where: C_A is the absorbance of TPF extract in the control; T_A is the absorbance of TPF extract in the test with different concentrations of the toxicants as single, binary and ternary mixtures.

Data analysis – determination of toxicity threshold

Inhibition of dehydrogenase activity from each toxicity assessment was converted to a 0 to 100 % scale as shown in equation [2]. Normalized responses were generated as the mean and standard deviation from triplicate measurements.

$$R = \left| 1 - \frac{T_A}{C_A} \right| \times 100 \qquad [2]$$

where R is the inhibition of dehydrogenase activity (%), C_A is the absorbance of the TPF extract in the control experiment, and T_A is absorbance of TPF extract in the test experiment at different concentrations of metal ion(s). Dose-response data obtained from the evaluation of the toxic effects of toxicants in their binary and ternary mixtures on the dehydrogenase activity of the bacteria were plotted and fitted with 2-parameter logistic function (equation [3]), to obtain the respective toxicity threshold (EC50), which is defined as the con-

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centrations of the toxicants that inhibited the dehydrogenase activity of the bacteria by 50 %. The dose-Response data for individual substances as well as the mixtures:

$$R = \frac{100}{1 + \left(\frac{x}{EC_{50}}\right)^{b}} \quad [3]$$

where x is the metal ion concentration, EC_{50} is the concentration of metal ion(s) that inhibited dehydrogenase activity by 50 % and b is the slope at EC_{50}.

Toxicity Index (TI)

The toxicity index (TI) of each mixture was calculated as the sum of the toxic units of all components of the mixture (Equation [4]).

$$T1 = \sum_{i=1}^{n} \frac{C_i}{EC_{50i}} = \sum_{i=1}^{n} \frac{\pi_i EC_{50mix}}{EC_{50i}} \quad [4]$$

where x is the metal ion concentration; EC_{50} is the concentration of metal ion(s) that inhibited dehydrogenase activity by 50 % and b is the slope at EC_{50}; C_i is the concentration of the ith component mixture at the EC_{50} of the mixture (EC_{50mix}), and EC_{50i} is the concentration of the ith component that elicited 50 % decrease in dehydrogenase activity when tested as an individual, n is the number of components in the mixture, and πi is the proportion of the i-th component in the mixture. TI = 1 represents additivity, TI > 1 represents antagonistic interactions, and TI < 1 describes a synergistic interaction (Boillot and Perrodin, 2008).

Isobolographic analysis

The estimated EC_{50} was subsequently used to determine isoboles and isobolographic analysis of the toxicity of binary mixtures, as described in (Nweke et al., 2014). Isobols were calculated using the concentrations of each component calculated in the EC_{50} (C_i). The C_i values (C_{iA} and C_{iB}) of the components can be calculated by multiplying the proportion of each component in the mixture by the EC_{50} of the mixture, as in the numerators of Equation 4. Triplicate isoboles were generated and plotted in isobologram as described elsewhere (Nweke et al., 2014). The straight line connecting the EC_{50} of component A on one axis and EC_{50} of component B on the other axis is the additivity line representing the additive effect of the mixture. The interaction is considered synergistic or antagonistic if the isobologram plotted on the isobologram lies below or above the additivity line, respectively. An interaction is considered additive if the isobologram plotted on the isobologram lies on the additivity line.

Results

Identification of the test organism. Identification of the isolates was based on their morphology, microscopic and biochemical characteristics. The isolate identified was *Leptothrix* sp.

Toxicity of Individual metal ions. The effects of the individual metal ion on the dehydrogenase activity of the *Leptothrix* sp. are shown in Figures 1, 2 and 3.

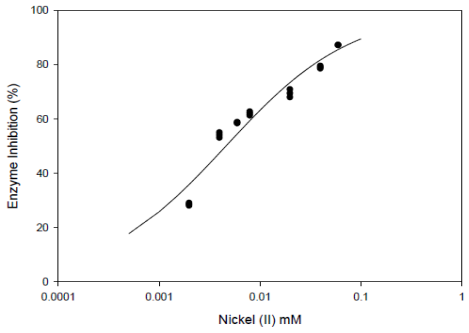

Figure 1 *Toxicity of nickel as individual chemical on dehydrogenase activity of Leptothrix sp.*

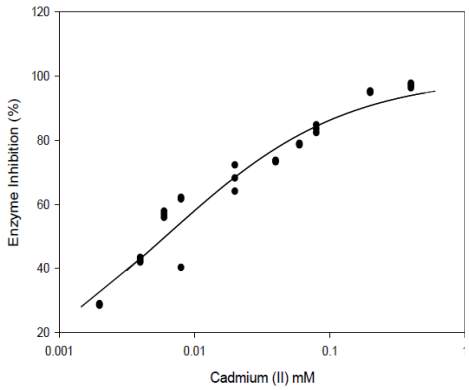

Figure 2 *Toxicity of cadmium as individual chemical on dehydrogenase activity of Leptothrix sp.*

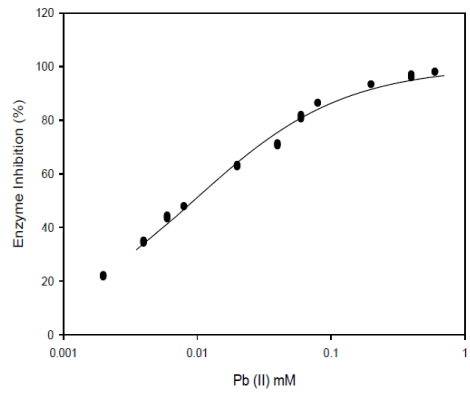

Figure 3 *Toxicity of lead as individual chemical on dehydrogenase activity of Leptothrix sp.*

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The response of the organism to the toxicity of the metal ions was dose-dependent. The metal ions progressively inhibited the dehydrogenase activity as the concentrations were increased, giving percent inhibitions greater than 95% at 0.2 mM Cd(II), 0.4 mM Pb (II). The percentage inhibition of Ni (II) was greater than 85% at 0.06 mM. The experimental data for all the metals were fitted with 3-parameter logistic model to obtain the dose-response curve. The shapes of the dose-response curves are rather similar, indicating the similarity of the molecular mechanisms of the actions of the metal ions. Nickel and cadmium exhibited sharp inhibitory effect as the concentration increases; lead showed a progressive inhibition of the dehydrogenase activity as the concen-. tration increases

Toxicity of binary mixtures. The toxicity of the binary mixtures was accessed using six different metal combinations in a fixed ray uniform design. Figure 4 and 5, shows the model fit curves of the experimental dose-response relationships for the assessment of toxic effects of binary mixtures of Cd (II) + Ni (II) and Pb (II) + Ni (II), on dehydrogenase activity of *Leptothrix* sp.
The dose-response patterns are describable with the 2-parameter logistic model. The binary mixture ratio of Cd (II) + Ni (II) and Pb (II) + Ni (II) shows inhibitory effect of the dehydrogenase enzyme activity as the concentration increases.

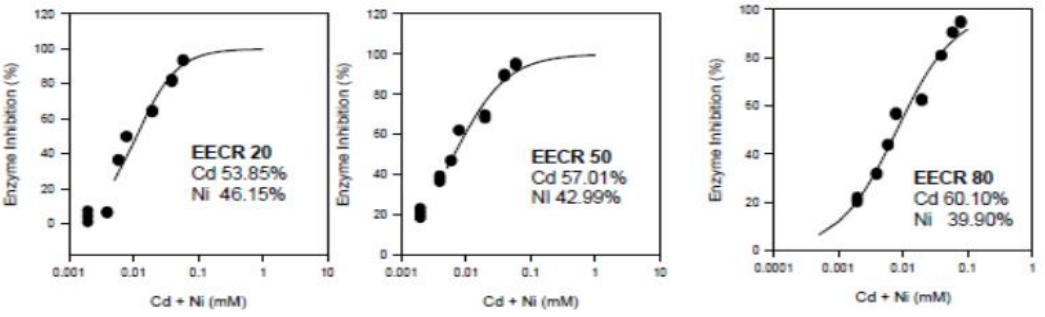

Figure 4
Toxicity of binary mixtures of cadmium and nickel ions on dehydrogenase activity of Leptothrix sp.

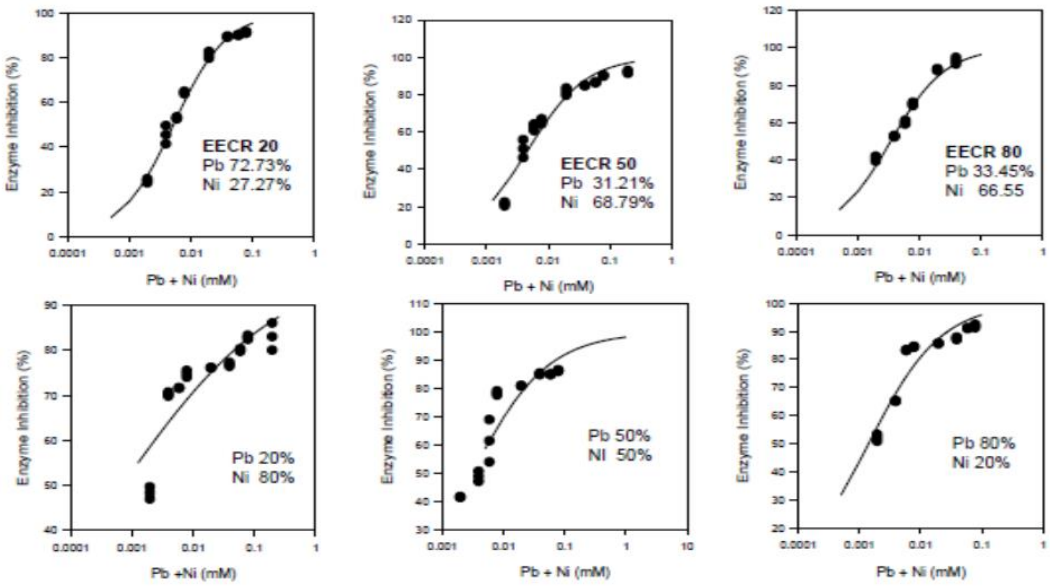

Figure 5
Toxicity of binary mixtures of lead and nickel ions on dehydrogenase activity of Leptothrix sp.

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Toxicity of ternary mixtures. The toxicity of the ternary mixtures was accessed using six different metal combinations in a fixed ray uniform design. Figure 6, shows the model fit curves of the experimental dose-response relationships for the assessment of toxic effects of ternary mixtures of Cd (II) + Pb (II) + Ni(II), on dehydrogenase activity of *Leptothrix* sp.. The dose-response patterns are describable with the 2- parameter logistic model. The ternary mixture ratio of Cd (II) + Pb (II) + Ni (II) shows inhibitory effect of the dehydrogenase enzyme activity as the concentration increases.

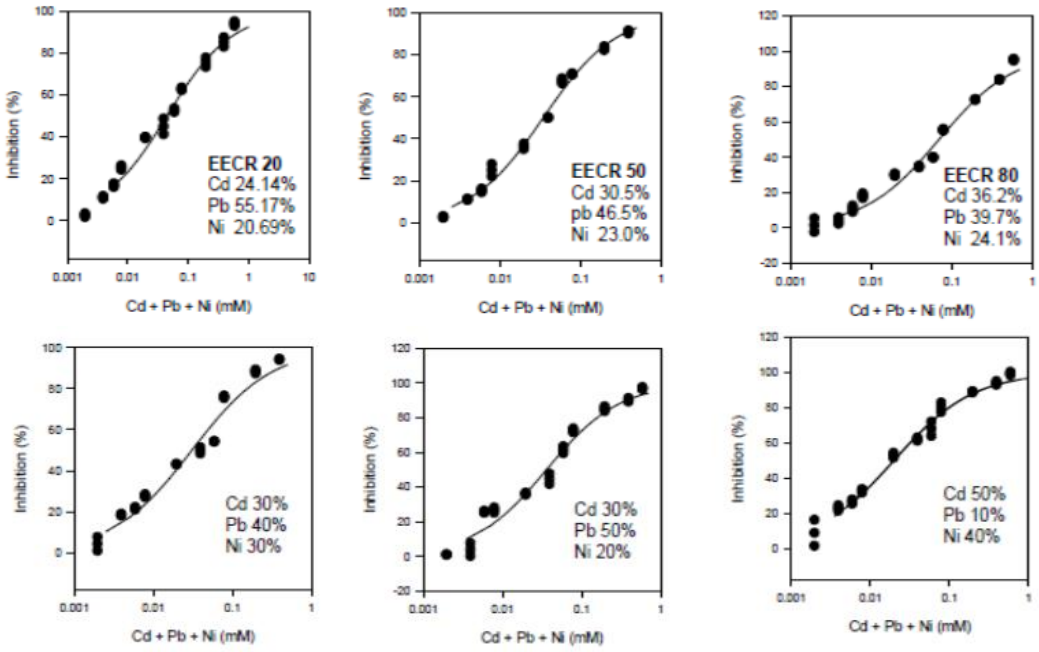

Figure 6
Toxicity of ternary mixtures of cadmium, lead and nickel ions on dehydrogenase activity of Leptothrix sp.

Toxicity threshold (EC_{50})

Toxicity threshold (EC_{50}) of Individual metal ions. The EC_{50} values of the metals are 0.0046mM for Ni(II), 0.0061 for Cd(II) and 0.0094 mM for Pb(II). The Duncan test indicates that the EC_{50} of the metals were significantly different from each other and the order of toxicity is Ni(II)> Cd (II)> Pb(II). The toxicity threshold, (EC_{50}) of all the single metals on the dehydrogenase enzyme activity of *Leptothrix* sp. is shown in Table 3.

Toxicity threshold (EC_{50}) of binary mixtures. The EC_{50} of the Pb (II) + Ni (II) mixtures ranged from (0.000676 + 0.000476) to (0.0052 + 0.00036) mM. It was observed that Ni (II) proved to be more toxic than Pb (II) as in the case of the mixture ratio of Pb (II) 72.73% + Ni (II) 27.27% (EECR-20); it had the highest concentration of Pb (II) and lowest concentration of Ni (II) which yielded the least toxicity value of (0.0052 + 0.00036) mM, whereas mixture ratios of Pb (II) 33.45% + Ni (II) 66.55% (EECR-80) and Pb (II) 31.21% + Ni (II) 68.79% (EECR-50) yielded higher toxicities due to increasing concentrations of Ni (II). Mixture ratios of both Pb (II) 20% + Ni (II) 80% had the highest toxicity value of 79 (0.000676 + 0.000476) mM followed by Pb (II) 80% + Ni (II) 20% irrespective of the equal varying combination of the metal ions . The EC_{50} of the mixtures were significantly different from each other and the order of toxicity is Pb (II) 20% + Ni (II) 80% > Pb (II) 80% + Ni (II) 20% > Pb (II) 50% + Ni (II) 50% > Pb (II) 33.45% + Ni (II) 66.55% (EECR-80) > Pb (II) 31.21% + Ni (II) 68.79% (EECR-50) > Pb (II) 72.73% + Ni (II) 27.27% (EECR-20). The EC_{50} of the Cd(II) + Ni(II) mixtures ranged from (0.0065 + 0.00054) to (0.0145 + 0.0005) mM. The EC_{50} of the mixtures were significantly different from each other and the order of toxicity is Cd (II) 57.01% + Ni (II) 42.99% (EECR-50)

Table 3. *Toxicity thresholds (EC_{50}) of chemicals as single metals on dehydrogenase activity of Leptothrix sp*

Singles	EC50 (mM)
Ni	0.0046±0.0008
Cd	0.0061±0.0009
Pb	0.0094±0.0005

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> Cd (II) 60.10% + Ni (II) 39.9% (EECR-80) > Cd (II) 50% + Ni (II) 50% > Cd (II) 53.85% + Ni (II) 46.15% (EECR-20) > Cd (II) 80% + Ni (II) 20% > Cd (II) 20% + Ni (II) 80%. Generally, the order of decrease in toxicities for the toxic interaction of the binary mixtures is Pb(II) + Ni(II) > Cd (II) + Ni (II). The toxicity threshold (EC50) of all the binary mixtures on the dehydrogenase enzyme activity of *Leptothrix* sp., are shown in Table 4.

Table 4
Toxicity thresholds (EC_{50}) of chemicals as binary mixtures on dehydrogenase activity of Leptothrix sp.

BINARY	EC_{50} (mM)
Pb (II)+ Ni (II)	
Pb (II) 72.73% + Ni (II) 27.27% (EECR-20)	0.0052±0.00036
Pb (II) 31.21% + Ni (II) 68.79% (EECR-50)	0.00459±0.0006
Pb (II) 33.45% + Ni (II) 66.55% (EECR-80)	0.00341±0.00026
Pb (II) 20% + Ni (II) 80%	0.000676±0.000476
Pb (II) 50% + Ni (II) 50%	0.00302±0.00067
Pb (II) 20% + Ni (II) 80%	0.00141±0.00043
Cd (II)+ Ni (II)	
Cd (II) 53.85% + Ni (II) 46.15% (EECR-20)	0.01117±0.0014
Cd (II) 57.01% + Ni (II) 42.99% (EECR-50)	0.0065±0.00054
Cd (II) 60.10% + Ni (II) 39.9% (EECR-80)	0.00804±0.0007
Cd (II) 20% + Ni (II) 80%	0.0145±0.0005
Cd (II) 50% + Ni (II) 50%	0.00985±0.0007
Cd (II) 80% + Ni (II) 20%	0.0129±0.0006

Toxicity threshold (EC_{50}) of ternary mixtures. The ternary mixture ratio of Cd(II) + Pb(II) + Ni(II) shows inhibitory effect of the dehydrogenase enzyme activity as the concentration increases. The EC_{50} of the Cd(II) + Pb(II) + Ni(II) mixtures ranged from (0.0199 + 0.0017) to (0.0696 + 0.0066) mM. It was observed that the mixture ratio of Cd (II) 50% +Pb (II) 10% +Ni (II) 40% had the highest toxicity of (0.0199 + 0.0017) mM and Cd (II) 36.2% +Pb (II) 39.7% +Ni (II) 24.1% (EECR-80) had the lowest toxicity of (0.0696 + 0.0066) mM. The concentration ratio of Pb(II) was relatively higher in all the mixture ratios except for Cd (II) 50% +Pb (II) 10% +Ni (II) 40% which had the highest toxicity and seems to be dependent on the relative amount of Cd(II) in the mixture.

The EC_{50} of the mixtures were significantly different from each other and the order of decrease in toxicity is Cd (II) 50% +Pb (II) 10% +Ni (II) 40% > Cd (II) 30% +Pb (II) 40% +Ni (II) 30% > Cd (II) 30.5% +Pb (II) 46.5% +Ni (II) 23.0% (EECR- 50) > Cd (II) 30% +Pb (II) 50% +Ni (II) 20% > Cd (II) 24.14% +Pb (II) 55.17% +Ni (II) 20.69% (EECR-20) > Cd (II) 36.2% +Pb (II) 39.7% +Ni (II) 24.1% (EECR-80). Generally, the order of decrease in toxici-ties for the toxic interaction of the ternary mixtures are Cd(II) + Pb(II) + Zn(II) > Cd(II) + Pb(II) + Co(II) > Cd (II) + Pb (II) +Ni (II). The toxicity threshold (EC50) of all the ternary mixtures on the dehydrogena-se enzyme activity of *Leptothrix* sp., are shown in Table 5.

Table 5
Toxicity thresholds (EC_{50}) of chemicals as ternary mixtures on dehydrogenase activity of Leptothrix sp.

TERNARY	EC_{50} (mM)
Cd (II)+ Pb (II)+ Ni (II)	
Cd (II) 24.14% +Pb (II) 55.17% +Ni (II) 20.69% (EECR-20)	0.0446±0.0036
Cd (II) 30.5% +Pb (II) 46.5% +Ni (II) 23.0% (EECR-50)	0.0343±0.0024
Cd (II) 36.2% +Pb (II) 39.7% +Ni (II) 24.1% (EECR-80)	0.0696±0.0066
Cd (II) 30% +Pb (II) 40% +Ni (II) 30%	0.0307±0.0039
Cd (II) 30% +Pb (II) 50% +Ni (II) 20%	0.036 ±0.005
Cd (II) 50% +Pb (II) 10% +Ni (II) 40%	0.0199±0.0017

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Toxic interactions of metal mixtures. The toxic indices and the effect of the binary and ternary mixtures on dehydrogenase activities are shown in Table 6 and 7. Generally, the binary mixtures of Pb(II) + Ni(II) was synergistic except for Pb (II) 31.21% + Ni (II) 68.79% (EECR-50) which was additive. The binary mixture of Cd (II) + Ni (II) was entirely antagonistic. The ternary mixtures were all antagonistic. This is because the various ratio concentrations of the metal mixtures in combination when compared to their individual degree of toxicity did not yield the expected toxic effects.

Table 6
Toxic Interactions of the binary mixtures of the test chemicals on dehydrogenase activity of the Leptothrix sp.

BINARY	Toxic Index (TI)	Interaction
Pb (II)+ Ni (II)		
Pb (II) 72.73% + Ni (II) 27.27% (EECR-20)	0.714±0.028	Synergistic
Pb (II) 31.21% + Ni (II) 68.79% (EECR-50)	0.840±0.002	Additive
Pb (II) 33.45% + Ni (II) 66.55% (EECR-80)	0.623±0.044	Synergistic
Pb (II) 20% + Ni (II) 80%	0.125±0.075	Synergistic
Pb (II) 50% + Ni (II) 50%	0.488±0.047	Synergistic
Pb (II) 20% + Ni (II) 80%	0.180±0.039	Synergistic
Cd (II)+ Ni (II)		
Cd (II) 53.85% + Ni (II) 46.15% (EECR-20)	2.116±0.069	Antagonistic
Cd (II) 57.01% + Ni (II) 42.99% (EECR-50)	1.226±0.096	Antagonistic
Cd (II) 60.10% + Ni (II) 39.9% (EECR-80)	1.502±0.102	Antagonistic
Cd (II) 20% + Ni (II) 80%	3.057±0.403	Antagonistic
Cd (II) 50% + Ni (II) 50%	1.899±0.164	Antagonistic
Cd (II) 80% + Ni (II) 20%	2.274±0.255	Antagonistic

Table 7
Toxic interactions of the ternary mixtures of the test chemicals on dehydrogenase activity of the Leptothrix sp.

TERNARY	Toxic Index (TI)	Interaction
Cd (II)+ Pb (II)+ Ni (II)		
Cd (II) 24.14%+Pb (II) 55.17%+Ni (II) 20.69% (EECR-20)	6.458±0.194	Antagonistic
Cd (II) 30.5% +Pb (II) 46.5% +Ni (II) 23.0% (EECR-50)	5.158±0.296	Antagonistic
Cd (II) 36.2% +Pb (II) 39.7% +Ni (II) 24.1% (EECR-80)	10.762±0.408	Antagonistic
Cd (II) 30% +Pb (II) 40% +Ni (II) 30%	4.834±0.028	Antagonistic
Cd (II) 30% +Pb (II) 50% +Ni (II) 20%	5.211±0.045	Antagonistic
Cd (II) 50% +Pb (II) 10% +Ni (II) 40%	3.625±0.224	Antagonistic

Isobolographic analysis of binary mixtures. The isobolographic analyses of the mixtures based on the EC_{50} values are shown in Figure 7. The isobologram indicated addictive effect for Pb (II) 31.21% + Ni (II) 68.79% (EECR-50). For the Cd (II) + Ni (II) mixtures, the isobologram indicated antagonistic effect for all the mixtures.

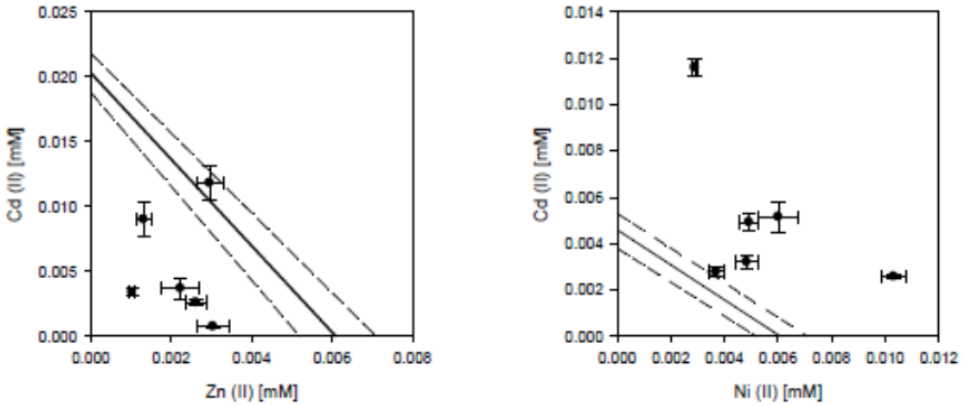

Figure 7
The EC_{50} isobole representations for metal ions as individual and mixtures tested against DHA of Leptothrix sp. The bars represent the standard deviations of the 95% confidence interval of the values. The solid and dotted lines represents additivity line and its 95% confidence belt

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Discussion

Leptothrix sp is an important environmental microorganism with a unique role in soil fertility. *Leptothrix* sp. is a sheathed filamentous bacterium commonly found in different types of aquatic/moist environments with sufficient organic matter. *Leptothrix* is known to be able to oxidize both iron (II) and manganese (II). The products of these oxidation reactions are ferric hydroxide and manganese oxide. They are well adapted to soil and are therefore being extensively studied for use in applications that require the release and survival of soil bacteria (Girija and Kumar, 2005). The toxic effects of binary and tertiary mixtures of nickel, lead and cadmium on *Leptothrix* sp using inhibition of dehydrogenase activity as the endpoint were evaluated. A uniform design and equieffect concentration ratios were used to design the experiment, exposing the organism to different concentrations of metal ions. The toxic index approach and isobolographic analysis indicated that interactions (synergistic, antagonistic and additivity) are possible between different mixtures of metals. Cadmium and lead have been reported to inhibit microbial populations and enzyme activity in soil and water environments. For example, increasing soil cadmium and lead concentrations have been reported to gradually reduce microbial communities and enzymes (Abdousalam, 2010; Xiao et al., 2020). Although Ni is a trace element, it is toxic to microorganisms at high concentrations. Nweke and colleagues reported heavy metal inhibition of dehydrogenase enzymes on purified cultures of bacteria and microbial communities in soil and river water (Nweke and Okpokwasili, 2012; Nweke and Orji, 2009). Cadmium has no physiological function and strongly inhibits microbial metabolism even at low concentrations. In the toxicity of individual chemicals, the results of this study showed that nickel and cadmium exhibited a sharp inhibitory effect with increasing concentration, while lead exhibited a gradual inhibition of dehydrogenase activity with increasing concentration. Similarly, Nweke et al. (2018) also reported that nickel and cadmium ions inhibit microbial activities at high concentrations. The EC_{50} of the individual chemicals in this study was recorded at 0.0046 ± 0.0008 mM for Ni, 0.0061 ± 0.0009 mM for Cd and 0.0094 ± 0.0005 mM for Pb. Toxicity threshold (EC_{50S}) of 0.080 ± 0.006 mM Ni has been reported against *Pseudomonas fluorescens* (Nweke et al., 2020). Okechi et al. (2020) also reported that the dehydrogenase activity of *S. marcescens* (SerEW01) from the river was inhibited by cadmium and lead at thresholds of 0.058 ± 0.002 mM and 0.113 ± 0.005 mM, respectively. Furthermore, EC_{50S} 0.023 ± 0.003 mM Cd and 0.135 ± 0.007 mM Pb were reported to inhibit dehydrogenase activity in the *Pseudomonas fluorescence* in soil (Nweke et al., 2020). The order of toxicity ranking of toxic substances is Cd > Ni > Pb (Mansour et al., 2015). The toxic effects of binary and tertiary mixtures of these metals were evaluated. Microbial species in soil and water environments are rarely exposed to single metal pollution, but rather a mixture of metals from anthropogenic sources in varying concentrations and concentration ratios. These metal mixtures interact and regulate each other's toxicity. The result of these metal interactions gives additive, synergistic or antagonistic interaction. The assessment of metal mixtures is the top priority for establishing tolerance levels for metals in the environment. Isobolographic analysis and toxicity index were used to characterize the joint effects of a binary mixture of Cd(II) + Ni(II) and Pb(II) + Ni(II). Both approaches show synergistic, antagonistic and additive effects for different mixture ratios, the same approach was also observed in the study done by Nweke et al. (2018) and Okechi et al. (2020). Tertiary mixtures of Cd(II) + Pb(II) + Ni(II) showed antagonistic effects for different mixing ratios. Several authors have reported both synergistic and antagonistic interactions in studies with tertiary mixtures of various heavy metals with bacteria and algae (Nweke and Orji, 2009; Cristani et al., 2011; Franklin et al., 2002).

Conclusion

Toxic effects of binary and tertiary mixtures of nickel, cadmium and lead on *Leptothrix* sp. The use of inhibition of dehydrogenase activity as the endpoint was evaluated. Uniform design and equieffect ratios were used to design experiments, exposing the organism to different concentrations of metal ions. The toxic index approach and isobolographic analysis indicated that the interactions; synergistic, antagonistic and additivity are possible among the different metal mixtures. The results of this study provide information on the possible synergistic interactions of metal mixtures. This raises concerns about the presence of mixed metals in the natural environment. This information will influence the development of environmental legislation regarding the tolerance of metals in the environment.

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