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Molecular Characterization and Toxicity of Heavy Metal Quinary Mixtures on *Enterobacter cloacae* Isolate from Nworie River

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The molecular characterization and toxicity of Cadmium (Cd), Lead (Pb), Zinc (Zn), Chromium (Cr) and Copper (Cu) quinary mixtures on *Enterobacter cloacae* isolate from Nworie River was investigated. Quinary mixtures of the heavy metals were compounded using fixed ratio (%); and inhibitory effect assessed using inhibition of total dehydrogenase as toxicity response. The mixtures consisted of five heavy metals in the ratios: Pb (20%) + Cr (20%) + Cd (20%) + Zn (20%) + Cu (20%), Pb (30%) + Cr (20%) + Cd (10%) + Zn (30%) + Cu (10%). Pb (10%) + Cr (10%) + Cd (30%) + Zn (40%) + Cu (10%), Pb (15%) + Cr (25%) + Cd (25%) + Zn (15%) + Cu (20%), Pb (40%) + Cr (15%) + Cd (5%) + Zn (20%) + Cu(20%). Result obtained showed that toxicity of the metals against the bacterium ranked in the order Cu > Cr >Zn > Pb > Cd. The quinary mixtures Pb (20%) + Cr (20%) + Cd (20%) + Zn (20%) + Cu (20%), Pb (30%) + Cr (20%) + Cd (10%) + Zn (30%) + Cu (10%) and 10%Pb + 10%Cr + 30%Cd + 40%Zn + 10%Cu showed synergistic interaction, while 15%Pb + 25%Cr + 25%Cd + 15%Zn + 20%Cu and 40%Pb + 15%Cr + 5%Cd + 20%Zn + 20%Cu mixtures were antagonistic and additive respectively. Threshold inhibitory

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concentration (IC_{50}) of the quinary mixtures was 0.054 ± 0.005mM, 0.053 ± 0.002 mM, 0.038 ± 0.002 mM, 0.077 ± 0.007 mM and 0.058 ± 0.006 mM for the respective mixtures. The toxic index values evaluated for the mixtures exhibited antagonistic, synergistic and additive interaction in the various ratios. The 16S rRNA and ITSF1 sequences obtained of the isolate showed the phylogenetic placement of the 16S rRNA of the isolate was closely related to *Enterobacter cloacae* strain DL01 (MH168084). The heavy metals mixtures demonstrated diverse toxicity interaction on the isolate depending on their relative composition, thus poses a threat to aquatic microbial diversity.

Keywords: Nworie River; quinary; mixture; toxicity; chromium; lead; cadmium.

1. INTRODUCTION

Heavy metals are metals with relatively high densities, atomic weights, or atomic numbers. They are relatively not so common in the lithosphere but are important in many parts of modern life. Metals are vital cellular components of all living systems. It has been reported that some of them are cofactors of enzymes that partake in crucial cellular processes while others are needed for the maintenance of inter and intracellular ionic balances [1]. However, heavy metals like cadmium, mercury, lead, and arsenic are highly poisonous. When heavy metals occur at high levels, they can cause adverse effects on the ecology of organisms and plants found in water bodies and may lead to a decline in their population [2]. Previous studies have reported deterioration of water quality with organic wastes [3]. Heavy metals like Zinc and Copper have roles to play in the physiological functions of microorganisms. Although at high concentrations they can cause inhibition of microbial processes. They are involved in redox processes which help to stabilize molecules as components of enzymes and for regulation of osmotic pressure. They also serve as micronutrients [4].

Nworie River is a major river in Owerri, Imo state. It covers a distance of about 7.5km across the Owerri geo-political zone in Southeastern Nigeria [5]. The River passes through three local government areas namely: Owerri North, Owerri Municipal, and Owerri West. According to Udensi *et al.,* [6], the Nworie river transverses the Owerri urban areas and is prone to intense industrial and human activities. Run-offs from towns and villages empty into the River thereby causing siltation and sedimentation that leads to water and soil pollution [7]. Heavy metals like Cadmium, Lead, Mercury, Silver, and Gold have biochemical functions and are toxic to living organisms [4]. The mechanism of toxicity of heavy metals to microorganisms ranges from blocking essential functional groups, displacing essential metal ions, modification of the active conformation of biomolecules, denaturing, and inactivating cell membrane integrity [8-9]. Industrialization and urbanization have prompted an increase in the discharge of wastes containing agrochemicals, petrochemicals, and heavy metals, organic and inorganic compounds into the environment posing life-threatening environmental pollution [10]. The bioaccumulation of heavy metals that are toxic in the biota of riverine ecosystems results in various adverse effects on humans and animals upon consumption of such water [11]. Natural and anthropogenic sources of heavy metal pollution of water bodies occur as suspended solids as well as sediments [12-13]. Determination of the toxicity of metals to bacteria is important for the evaluation of pollution risk in the environment. Recent researches have focused on developing rapid and sensitive assays for the assessment of toxicity using microbes and microbial enzymes. In the present study, the toxicity of Cadmium, Lead, Zinc, Chromium, and Copper quinary mixtures on *Enterobacter cloacae* isolate from Nworie River was assessed to elucidate the toxicity and interaction effect of the metal mixtures using fixed ratios.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Water samples were collected randomly from various points along the course of Nworie River located at Egbeada, Owerri North L.G.A. of Imo State, Nigeria. Water samples were collected along the course of the river at two spots (N 5° 31'2.934", E 7° 0'57.348", N 5° 30'56.586", E 7° 0'59. 532" at a depth of about 1 meter and pooled into sterile sample bottles. The samples were placed in an ice pack and transported to the laboratory for analysis.

2.2 Isolation of Organisms

Ten-fold serial dilution of the water samples as described by Ogbulie *et al.,* [14] was carried out

using physiological saline (0.85% NaCl) as diluents. A 0.1ml aliquot of the dilution was inoculated onto nutrient agar plates and incubated for 24 hours at room temperature (28±2°C). Different colonies developed were further purified by sub-culturing on Nutrient agar plates respectively. Thereafter, the purified bacterial isolates were stored at -4°C until required for identification and toxicity studies.

2.3 Molecular Identification

Molecular identification was carried out on the isolate which involved DNA extraction, DNA quantification,16S rRNA amplification, Internal transcribed spacer (ITS) Amplification, and Sequencing.

2.3.1 DNA extraction

Bacterial DNA was extracted as described by Jackson *et al.,* [15]. Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 minutes. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 20 minutes. The heated bacterial suspension was cooled on ice and spun for 3 minutes at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml micro-centrifuge tube and stored at -20^0C for analysis.

2.3.2 DNA quantification

The Nanodrop 1000 spectrophotometer was used to quantify the genomic DNA that was extracted. Launching of the software of the equipment was done by double-clicking on the Nanodrop icon. Initialization and blanking of the instrument were done using 2µl of sterile water and normal saline respectively. The lower pedestal was loaded with 2µl of the extracted DNA, the upper pedestal was then brought down to the lower pedestal, to make contact with the extracted DNA. Measurement of the DNA level was done by a click on the “measure” button.

2.3.3 16S rRNA amplification

The 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers were used to amplify the 16s region of the rRNA genes of the isolate on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. In the PCR mixes were: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4M concentration of the primers (F 20bp, R 18bp), and the template containing the extracted DNA. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The 1.5Kb amplicons were resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.3.4 Internal transcribed spacer (ITS) AMPLIFICATION

The ITS region of the rRNA genes of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3 primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.5µM, and the extracted DNA as a template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

2.3.5 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition was as follows 32 cycles of 96°C for 10 sec, 55°C for 5 sec, and 60°C for 4min.

2.3.6 Phylogenetic analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN. These sequences were aligned using ClustalX. The evolutionary

history was inferred using the Neighbor-Joining method in MEGA 6.0 [16]. The bootstrap consensus tree inferred from 500 replicates [17], is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [18].

2.4 Preparation of Stock Metal and Mixtures

The stock metal solutions each of $CuCl_2.2H_2O$, $CdSO_4$, K_2CrO_7, $PbNO_3$ and $ZnCl_2$ was prepared to obtain a solution of 10mM stock in sterile deionized water. The quinary mixtures of lead, chromium, cadmium, zinc and copper was compounded in-situ with a common stock concentration of the single metals using fixed ratio rays experimental design. This was designed as described by Nweke *et al.,* [19]. The quinary mixtures consisted of the five heavy metals in the ratios: Pb(20%) + Cr(20%) + Cd(20%) + Zn(20%) + Cu(20%), Pb(40%) + Cr(15%) + Cd(5%) + Zn(20%) + Cu(20%), Pb(15%) + Cr(25%) + Cd(25%) + Zn(15%) + Cu(20%), Pb (10%) + Cr(10%) + Cd(30%) + Zn(40%) + Cu(10%), Pb(30%) + Cr(20%) + Cd(10%) + Zn(30%) + Cu(10%).

2.5 Toxicity of Heavy Metal and Mixtures

The toxicity of single metals and quinary mixtures of the five metals (lead, chromium, cadmium, zinc, and copper) was assessed using fixed ratio rays experimental design as described by Nweke *et al.,* [19]. Inhibition of total dehydrogenase activity was determined using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as the artificial electron acceptor which was reduced to the purple-colored formazan crystals. The inhibition of dehydrogenase activity was determined by using an appropriate volume of nutrient broth (pH 7) and MTT supplemented with varying concentrations of heavy metals or mixtures. A 0.5ml portion of 4-fold strength nutrient broth and requisite volumes of sterile deionized water and stock solution (10mM) of respective toxicants was added to each 20ml screw-capped test tube to obtain the different single and complex mixture set-up of the heavy metals. Thereafter, 0.2mls each of 0.1%w/v solution of MTT and bacteria isolate suspension was added into each tube. The controls consisted of the medium without the toxicants. The set-ups were incubated at room temperature (28 ± 2^0C) for 24 hours. Thereafter, the formazan crystals produced in each tube were extracted in 4ml of butanol. Absorbances of the extracts was determined spectrophotometrically at 500nm.

2.6 Data Analysis

The percentage inhibition of dehydrogenase activity of the isolate was calculated relative to control using equation 1 as described by Nweke *et al.,* [20]. The inhibition responses were generated as mean and their standard deviations from triplicate determinations.

$$R = \left(1 - \frac{TA}{CA}\right) \times 100 \quad (1)$$

Where R is the inhibition (%) of dehydrogenase activity, CA is the absorbance of the extracts in the control experiment and TA is the absorbance of the extract in the test experiment with different concentrations of metal ion (s).

The dose-response data of the single toxicants as well as their mixtures were then plotted and fitted with a 2-parameter logistic function as shown in equation 2.

$$R = \frac{100}{1 + \left(\frac{x}{EC_{50}}\right)^b} \quad (2)$$

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

2.7 Toxic Index

The toxic index (TI) of each mixture was calculated as the sum of toxic units for all mixture components. (Eq 3 & 4)

$$TI = \sum_{i=1}^{n} TU_i \quad (3)$$

$$TU_i = \frac{Cmix_i}{IC_{50I}} \quad (4)$$

Where C_{mixi} is the concentration of the ith toxicant in the mixture and IC_{50i} is the IC_{50} of the same toxicant when tested as a single metal. TI=1 signifies additive interaction, TI > 1 signifies antagonistic interaction and TI < 1 signifies synergistic interaction [21].

3. RESULTS AND DISCUSSION

3.1 Molecular Characterization

The 16s rRNA and ITSF1 sequences obtained from the isolates produced a similar match in the course of the mega blast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA and ITSF1 of the isolates B1, B2, B3, B4, and F1 respectively showed a percentage similarity to other species at 99%-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate to be *Enterobacter sp,* which was revealed to be closely related to *Enterobacter cloacae* strain DL01(MH168084), than *Enterobacter sp.*

3.2 Toxicity of Single Metals to *Enterobacter cloacae*

Results of the model fitted curve for the assessment of toxic effects of the single metals against *Enterobacter cloacae* are presented in Fig. 1. The sigmoidal curve associated with inhibitory effects of the metals showed progressive inhibition of the metals to the dehydrogenase activity of the organism. Copper showed the highest toxicity with total inhibition of the enzyme activity of the organism at 0.1 mM. This was followed by chromium with a total inhibitory concentration of 0.2 mM. Zinc and lead had relatively similar toxicity with total inhibition occurring at 0.4 mM each. Cadmium was the least toxic metal with total inhibition of the dehydrogenase activity at 1.5 mM. The toxicity of the individual metals against the bacterium ranked in the order Cu > Cr > Zn > Pb > Cd.

3.2.1 Toxicity of metal mixtures on dehydrogenase activity of *Enterobacter cloacae*

The results of the toxicity of the metal mixtures at different combination ratios to dehydrogenase activity of *Enterobacter cloacae* showed a progressive inhibition of total dehydrogenase activity of the bacterium with increasing concentrations. However, the mixture Pb(15%) + Cr(25%) + Cd(25%) + Zn(15%)+ Cu(20%) demonstrated low dose stimulation of dehydrogenase activity. Furthermore, beyond 0.1mM of the mixtures there was saturation and a declined toxicity of the mixtures on the isolates. The responses of the isolates to the mixtures Pb (20%) + Cr(20%) + Cd(20%) + Zn(20%) + Cu(20%), Pb(10%) + Cr(10%) + Cd(30%) + Zn(40%) + Cu(10%) and Pb(15%) + Cr(25%) + Cd(25%) + Zn(15%)+ Cu(20%) were closely describable by a sigmoidal relationship; while Pb(40%) + Cr(15%) + Cd(5%) + Zn(20%) + Cu(20%) and Pb(30%) + Cr(20%) + Cd(10%) + Zn(30%) + Cu(10%) were logistic.

3.3 Toxicity Thresholds

The 24-hrs toxicity thresholds (IC_{50}) of the toxicants as singles as well as mixtures are shown on Table 1. Copper was the most toxic heavy metal to *Enterobacter cloacae* with lowest IC_{50} value of 0.035 ± 0.005mM while cadmium was the least toxic metal with highest value of 0.208 ± 0.015mM. The IC_{50} values were significantly different ($P<0.05$); the pattern of toxicity of the metals to *Enterobacter cloacae* was Cu > Zn > Cr > Pb > Cd. Furthermore, IC_{50} obtained for the mixtures showed that 10%Pb + 10%Cr + 30%Cd + 40%Zn + 10%Cu was the most toxic mixture ratio evaluated with lowest IC_{50} values of 0.038 ± 0.002mM. However, there was no significant difference ($p>0.05$) in the toxic effect of Pb (20%) + Cr(20%)+ Cd(20%) + Zn(20%) + Cu(20%), Pb (40%) + Cr(15%) + Cd(5%) + Zn(20%) + Cu(20%) and Pb(30%) + Cr(20%) + Cd(10%) + Zn(30%) + Cu(10%) to *Enterobacter cloacae*. While the toxicity threshold of Pb (10%) + Cr(10%) + Cd(30%) + Zn(40%) + Cu(10%) and Pb (15%) + Cr(25%) + Cd(25%) + Zn(15%) + Cu(20%) mixtures were significantly different ($P<0.05$).

3.4 Toxic Interactions of the Metal Mixtures

The results of the toxic interactions of the metal mixtures according to toxic index (TI) analysis are shown in Table 2. The results show that antagonistic, synergistic and additive effects are exhibited by the various ratios evaluated. The ratios of Pb (20%) + Cr(20%) + Cd(20%) + Zn(20%) + Cu(20%), Pb(30%) + Cr(20%) + Cd(10%) + Zn(30%) + Cu(10%) and Pb(10%) + Cr(10%) + Cd(30%) + Zn(40%) + Cu(10%) showed synergistic interaction, and antagonistic effect to the enzyme activity of *E. cloacae*. However, the ratios of 40%Pb + 15%Cr + 5%Cd + 20%Zn + 20%Cu and Pb (15%) + Cr(25%) + Cd(25%) + Zn(15%) + Cu(20%) were additive and antagonistic respectively. The TI values ranged from 0.587 to 1.141 for the *E. cloacae*.

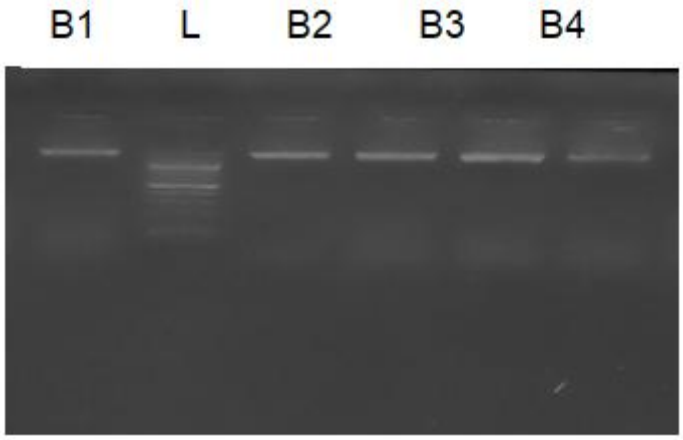

Plate 1. Agarose gel electrophoresis of the 16S rRNA gene of some selected bacterial isolates. Lanes B1-B5 represent the 16SrRNA gene bands (1500bp), lane L represents the 100bp molecular ladder

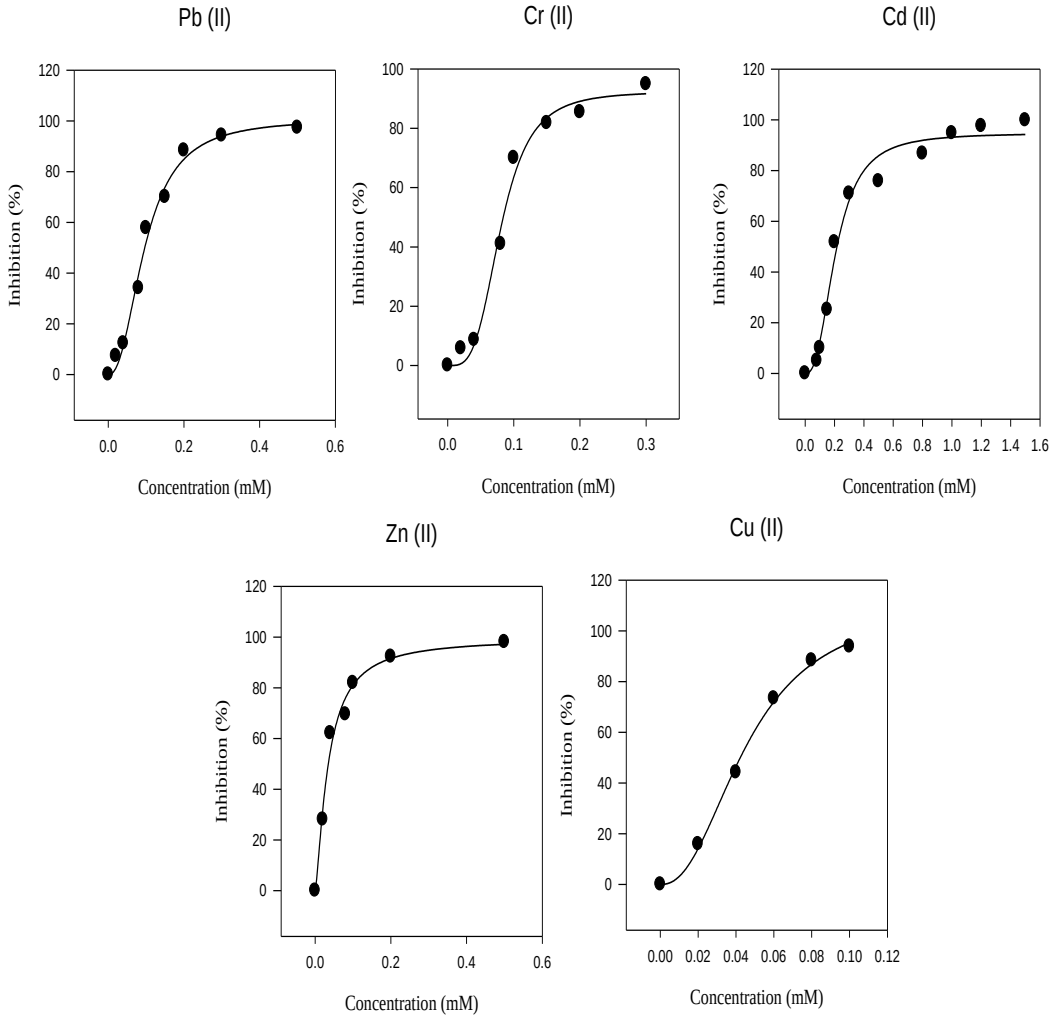

Fig. 1. Toxicity of lead, chromium, cadmium, zinc, and copper ions on dehydrogenase activity of *Enterobacter cloacae.*

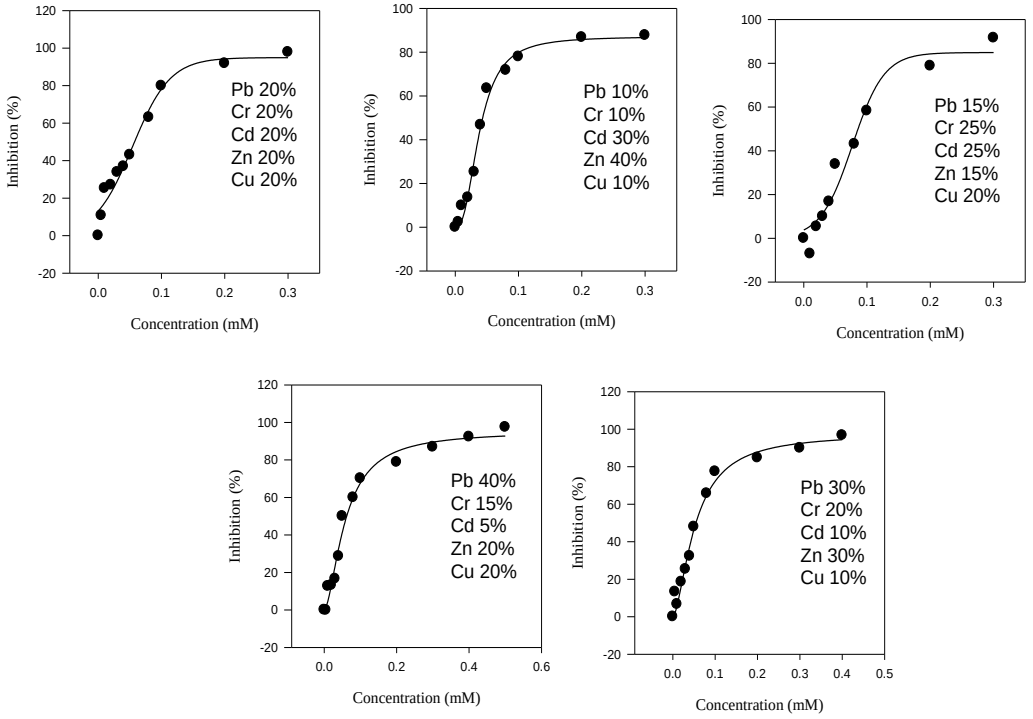

Fig. 2. Toxicity of lead, chromium, cadmium, zinc, and copper ions quinary mixtures on dehydrogenase activity of *Enterobacter cloacae*

Table 1. Toxicity threshold (IC_{50} mM) of the single and heavy metal mixtures to total dehydrogenase activity of the isolates

Heavy Metals	IC_{50} *of Enterobacter cloacae (*mM)
Lead	0.097± 0.006[a]
Chromium	0.080± 0.004[b]
Cadmium	0.208± 0.015[c]
Zinc	0.046± 0.003[d, e]
Copper	0.035± 0.005[e]
Mixtures	
20%Pb +20%Cr + 20%Cd + 20%Zn + 20%Cu	0.054± 0.005[a]
40%Pb + 15%Cr + 5%Cd + 20%Zn + 20%Cu	0.058± 0.006[a]
30%Pb + 20%Cr + 10%Cd + 30%Zn + 10%Cu	0.053± 0.002[a]
10%Pb+10%Cr +30%Cd+40%Zn+ 10%Cu	0.038± 0.002[b]
15%Pb + 25%Cr + 25%Cd + 15%Zn + 20%Cu	0.077± 0.007[c]

Values are mean ± standard deviation of triplicate determinations. Values of single and quinary mixtures with different superscripts are significantly different (P<0.05).

Table 2. Toxic interactions of the metal mixtures to dehydrogenase activities of *Enterobacter cloacae* isolates

Metal mixtures	TI values	R-value	Effect
20%Pb + 20%Cr + 20%Cd + 20%Zn + 20%Cu	0.845	0.9845	Synergistic
40%Pb + 15%Cr + 5%Cd + 20%Zn + 20%Cu	0.953	0.9828	Additive
30%Pb + 20%Cr + 10%Cd + 30%Zn + 10%Cu	0.819	0.9919	Synergistic
10%Pb + 10%Cr + 30%Cd + 40%Zn + 10%Cu	0.587	0.9945	Synergistic
15%Pb + 25%Cr + 25%Cd + 15%Zn + 20%Cu	1.141	0.996	Antagonistic

The present study investigated the toxicity of heavy metal quinary mixtures on *Enterobacter cloacae* isolate from the Nworie River. Heavy metals generally exert toxicity in microorganisms through blocking essential functional groups, displacing essential metal ions, or modifying the active conformation of biomolecules, denaturing and inactivating enzymes, and disrupting cell membrane integrity [9]. Our study revealed the susceptibility of the isolate dehydrogenase enzyme to the heavy metals toxicity both as single compounds and mixtures. Previous studies have reported the toxicity of heavy metals on pure cultures of bacteria and the microbial community of soil and river water [22-26]. Heavy metals are applied in various industrial processes; their pollution can result from either single or complex mixtures. In this study, Cu^{2+} ions were the most toxic heavy metal to the dehydrogenase enzyme activity of the bacterium with total inhibition of the enzyme activity of the organism occurring at 0.1 mM. This agrees with the findings of Nweke and Okpokwasili [26], on heavy metal toxicity threshold to *Pseudomonas* species from petroleum refinery wastewater; and the findings of Zolgharnein *et al.,*[27]. Zn and Pb had relatively similar toxicity with total inhibition occurring at 0.4 mM each. Reports have it that zinc inhibits microbial community diversity when in high concentrations, except for few resistant bacteria [28]. This is in line with reports from the study carried out by Nweke *et al.,* [19] whose findings have it that a 50% toxicity threshold of 0.180±0.010mm was recorded against *Pseudomonas fluorescens* dehydrogenase activity. However, in this study it was observed that Cadmium showed the least toxicity; the lower inhibitory effect of the cadmium on *E. cloacae* could be attributed to the high tolerance of the bacteria to the heavy metal. From the study, the different mixture ratios of heavy metals evaluated showed progressive inhibition of the enzyme activity of the bacterium as their concentrations increased. This is in line with the findings of Mani and Kumar [29] that all metals have the potential to exhibit harmful effects at higher concentrations and the toxicity of each metal depends on the amount available to the organism, the absorbed dose, the route, and the duration of exposure. The pattern of toxicity of the metals to *E. cloacae* ranked in the order, Cu > Zn > Cr > Pb > Cd. The analysis of IC50 values indicated that there is no significant difference in the toxic effect of the quinary mixtures. These suggest that the toxicity of the quinary mixtures is independent of the metal ion ratio composition of the mixtures. However, the toxic effect of the mixtures was monotonic but showed saturation above a threshold level for 15%Pb + 25%Cr + 25%Cd + 15%Zn + 20%Cu mixture. Toxic index analysis of the joint effects of the mixtures indicated that the combination of the metals modulates their toxicity. The interactions of the metal mixtures according to toxic index (TI) analysis showed synergistic, additive, and antagonistic effects as exhibited by the various ratios evaluated on E. cloacae. However, the synergistic interaction of the quinary mixtures was seen in mixtures with a high composition of Zn2+ ions. Mixture ratio-dependent effects have been reported for binary and quaternary mixtures of Ni (II) + Co(II) + Zn(II) + Cd(II)) on *P. fluorescens*; Ince *et al.,* [30] reported the same on the binary mixtures of heavy metal ions against *Vibrio fischeri* and *Lemna minor*. The synergistic effect implies that the combined effect of the metals is greater than the sum of the effects of each metal given alone. This closely agrees with our findings on the toxicity of the pattern of the metals singly to *Enterobacter cloacae* to be in the order Cu > Zn > Cr > Pb > Cd. Various statistical approaches have been employed to determine the combined effect of heavy metal toxicity at different concentration gradients; Fulladosa *et al.,* [31] reported the additive and antagonistic effect of Cd+ Cu, Cd +Pb, and Cu + Pb. Toxic Index analysis has been widely used in ecotoxicological studies to determine interactions of the mixture of toxicants [19, 21, 32-34].

4. CONCLUSION

The present study assessed the toxicity of heavy metal quinary mixtures on *Enterobacter cloacae* isolate from the Nworie River. The experimental results indicated that the five quinary mixture ratios investigated exhibited synergistic, additive, and antagonistic interactive effects on *E. cloacae* isolates. The study provides information on the potential synergistic toxicity of quinary mixtures with a relatively higher ratio of Zinc ions. The heavy metals mixtures demonstrated diverse toxicity interaction on the isolate depending on their relative composition, thus poses a threat to *E. cloacae* and in extension aquatic microbial diversity. Further research is recommended to ascertain the toxicity response of other microbial communities of Nworie River to forestall the degrading effect of anthropogenic activities on the water quality and aquatic life.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is 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 the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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