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Immobilization of Heavy Metals in Contaminated Soil using Calcium Phosphate Amendment and Phosphate-solubilizing Fungi (Penicillium sp) in Benin City, Edo state, Nigeria

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ABSTRACT: Soil is a limited resource that is continually contaminated by the presence of heavy metals as a result of human activities. This study used a combination of calcium phosphate amendment and phosphate-solubilizing fungi to immobilize heavy metals in polluted soil. Bovine bones were collected at an Abattoir Market in Aduwawa, Benin City, Nigeria with coordinates 6°22'6.24" N and 5°41'0.24" E and calcined to extract calcium-phosphate (hydroxyapatite), serial dilution and plating on Pikovskaya medium were utilized to obtain fungi extract from fertile soil. Prior to remediation, heavy metal analysis of polluted soil indicated high value of metal concentrations zinc 120.60mg/kg, chromium 22.67 mg/kg, cadmium 11.23 mg/kg, lead 18.43 mg/kg, iron 187.27 mg/kg and nickel 10.62 mg/kg above the World Health Organization permissible limit. There was a decrease in zinc and iron contents after the first week of treatment. The second week demonstrated a significant decrease in all metal concentrations; 27.53% for Zn, Cr 39.83%, Cd 46.13%, Pb 7.60%, Fe 46.26% and Ni 15.07% – indicating the treatment's effectiveness.

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The soil is the foundation of life on earth. A major portion of organisms live around the top 10 inches of the soil. By definition, soil is a biologically active complex mixture of weathered minerals, organic matter, organisms, air and water (Brady and Weil, 2002). This mixture performs a range of critical functions such as; supporting terrestrial ecosystems, biological diversity, agricultural food production, flood alleviation, water filtration, storage and carbon capture. Soils form over a long period of time should be considered a finite resource to be managed carefully (EPA 2021). In order to function properly, a stable soil must have constant supply of energy including nutrition both from abiotic and biotic sources (Ellwood and Dawkins, 2015). Soil possesses several properties that enables it carry out its function. The composition, shape, permeability, chemistry, bioactivities and color of the soil are all determined by the sum of these properties (Tolgyessy, 1993). Soil properties ensure soil health and are classified into chemical, physical and biological properties. The emergent problem regarding soil is pollution. Soil pollution is the introduction of harmful objects, chemicals or substances, directly or indirectly into soil in a manner which causes harm to living things or destroys the soil or water ecosystems (Bech *et al.*, 2014). Soil is often used as a sink for dumping solid and liquid wastes since urbanization began. The pollutants were thought to pose no danger to human health or the atmosphere when buried and out of reach, and that they would eventually vanish (Swartjes, 2011). Overpopulation has left a legacy of polluted soils around the world as

a result of industrialization, conflicts, logging, and intensified agricultural activities (Bundschuh *et al*., 2012; Liu and Li, 2015). The presence of some contaminants can cause nutrient imbalances and soil acidification, which are major issues in many parts of the world (FAO and ITPS, 2015). The contamination of soil by heavy metals has become a concerning issue in recent times (Hussain *et al.,* 2017). The most common heavy metals found at contaminated sites, in order of abundance are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu) and mercury (Hg) (US EPA, 1997). Heavy metals in soil come from a variety of sources which are broadly classified as natural or anthropogenic. Natural sources include volcanic eruptions, sea-salt sprays, forest fires, rock weathering, etc. and anthropogenic sources include industrial discharges, agricultural activities, mining and metallurgic operations, etc. (Masindi and Muedi, 2018). The availability of heavy metals in soil is affected by soil pH, organic matter concentration, presence of hydrous ferric oxide (Yi *et al.,* 2007), density and type of charge on colloids, degree of complexation with ligands and relative surface area of soil (Sharma and Raju, 2013). Heavy metals in soil are an increasing cause for concern, even in Nigeria, because they have the potential to reduce crop yields and the possibility of bioaccumulation and biomagnification in the food chain. Also, there is the risk of contamination of the surface and groundwater, as well as a variety of negative health effects on humans (Wuana and Okiemien, 2011). Heavy metals present risks to humans, animals, plants and ecosystems as a whole through processes such as direct ingestion, absorption by plants, food chains, consumption of contaminated water and alteration of soil pH, porosity, colour and its natural chemistry which in turn impact on the soil quality (Masindi and Muedi, 2018). The release of heavy metals into the air may result in health problems including skin and eye irritation, respiratory infections and cardiovascular problems, while causing environmental issues such as formation of acid rain, eutrophication and hazes (Chibuike and Obiora, 2014). Due to their negative effects on human and environmental health, various remediation technologies have been employed to control heavy metals in soil. A cost-effective method is the immobilization by use of organic adsorbents. Organic soil amendment can improve the soil properties for plant growth by providing nutrients as well as immobilizing heavy metals (Udeigwe *et al.,* 2011). Heavy metals can be immobilized by sorption and precipitation with soil amendments. It has been reported that bark, chitosan, zeolite, clay, fly ash, rice bran, and peat moss are some examples of low-cost adsorbents for heavy metal immobilization (Babel and Kurniawan, 2003). Although organic adsorbents do not remove heavy metals from the soil, they can reduce the mobility and dispersion of heavy metals (Lee *et al.,* 2013). The aim of this study was to evaluate the effectiveness of heavy metal immobilization in contaminated soil using a combination of calcium phosphate amendment and phosphate solubilizing fungi.

MATERIALS AND METHODS

Study Area: This study was carried out in Benin City, Edo state, Nigeria. It is located at latitude 06°19**'**00**"**E to 6°21'00"E and longitude 5°34'00"E to 5°44'00"E, in the tropical rainforest belt of southern Nigeria.

Sample Collection and Preparation: Old cow bones (bovine) were obtained from an Abattoir Market in Aduwawa, Benin City, Edo state with coordinates 6°22'6.24" N and 5°41'0.24" E. Bovine bones were washed with water and sodium carbonate to remove impurities and were sundried. The dried bovine bones were crushed into smaller pieces with a mallet and pulverised to powder. For the extraction of calcium phosphate from the bones, thermal decomposition was used. Ten (10) grams of pulverised bones were placed in an open alumina crucible and heated in a muffle furnace at 900 °C for 3 hours with heating rate of 10 °C/min to eliminate organic constituents in the bovine bone. Six rhizosphere soil samples with a greater portion of fertile soil containing microorganisms were randomly collected from a Banana and Yam plantation at University of Benin, Benin City, Edo state with coordinates 6°24'15"N, 5°37'50"E and 6°24'14"N, 5°37'55"E respectively while a contaminated soil sample were obtained from a Mechanical workshop at Federal College Road Osasogie, Benin City, Edo state with coordinates 6°23'14.8" N and 5°37'00.0" E. The samples were collected as follows: surface litter was scrapped off using a spade. An auger was driven into the soil at 0 - 10 cm depth. Obtained samples were placed in a labelled seedling bag. Upon arrival at the laboratory, the samples were air dried at 25°C - 35°C. Soil samples were sieved with 2mm mesh.

Analytical Procedure: Fungal species were isolated from soil samples collected from plant root zones using Pikovskaya's (PKV) agar medium. PKV medium was autoclaved at 121◦C for 15 minutes. 20 mL of the sterilized molten agar medium was poured into each petri dish and supplemented with 25μg/mL chloramphenicol to inhibit bacterial growth and allowed to solidify before inoculation. Ten grams of soil samples were aseptically weighed and transferred to 250 mL Erlenmeyer flask containing 90mL of 0.85% saline solution. The suspension was shaken on 110 rpm for 25 minutes on a rotary shaker and then allowed to settle for 10min. Aliquots of 1mL of the

supernatant from the sample was transferred to 9mL of sterile normal saline solution dispensed into test tubes and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Aliquots of 0.1mL were transferred and spread plated on Pikovskaya's agar plates and incubated at 28°C for 2 to 7days. Fungal colonies showing clear zones around the colonies were further purified by transferring into Pikovskaya's agar medium. Pure cultures were checked from nutrient agar plates. After achieving a pure culture, the same colony was streaked onto a nutrient agar slant. These cultures were incubated at 37 °C for 24 hours. They were then identified using Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The pH, organic matter content and particle size distribution of the contaminated soil sample were determined as follows: pH was determined using a pH meter, organic matter was determined using the Walkley and Black's method and the hydrometer method was used to determine the particle size distribution. Heavy metal analysis of the contaminated soil sample was carried out before the treatment and then for a further two weeks at one week interval after treatment was applied. One (1) gram of oven-dried soil sample was weighed and placed in a 250mL beaker previously washed with nitric acid and distilled water. To the soil, 5mL of HNO_3, 15mL of concentrated H_2SO_4 and 0.3mL of $HClO_3$ were added. The mixture was digested in a fume cupboard for 15 minutes after which it was cooled and diluted with distilled water. The mixture was filtered through Whatman filter paper into a 50mL volumetric flask and filled up to mark with distilled water. The sample was analysed using atomic absorption spectrophotometry (AAS) and the results were recorded in mg/kg. Calcium phosphate and the isolated phosphate solubilizing fungi were added to samples of heavy metal-contaminated soil. Calcium phosphate to soil ratio was 3:5. The mixture was kept and turned at intervals. At 7 days intervals, samples were taken from the inoculated soils and analysed for heavy metals to find out the effectiveness of immobilization. The entire process was carried out for 14 days and its efficiency calculated with the following formula after Zeng *et al*. (2020).

$$\boldsymbol{I} = \frac{a-b}{a} \text{ x } 100\%$$

Where, I = immobilization efficiency, a = initial extractable heavy metal value (mg/kg) and b = final extractable heavy metal value (mg/kg)

RESULTS AND DISCUSSION

Fertile soil samples used for the isolation of fungi species yielded the following phosphate solubilizing fungi: *Penicillium* sp. were observed in both Banana and Yam plantation soil samples. *Aspergillus* sp. were found in Yam plantation while *Fusarium* sp. appeared in only Banana plantation soil samples respectively, as shown in Table 1. *Penicillium* sp. were used to carry out this experiment due to its abundance in soil. The physicochemical properties of contaminated soil were recorded before analysis and are shown in Table 2. The particle size distribution showed the soil content as sand 88.71%, Clay 3.01% and silt 8.25 %. The average level of organic matter was 16.10 g/kg and it had an acidic pH of 3.56.

Table 1: Distribution pattern of fungi in soil samples

Source	Fungi species		
	Penicillium sp.	*Aspergillus* sp.	*Fusarium* sp.
Banana plantation	+		+
Yam plantation	+	+	

Table 2: Physicochemical analysis of contaminated soil

PARAMETERS	VALUES
Sand	88.71 %
Clay	3.01%
Silt	8.25 %
Organic Matter	16.10 g/kg
pH	3. 56

The results of heavy metal analysis of the contaminated soil sample before and after treatment with calcium phosphate and *Penicillium* sp. are shown in Table 3.

Table 3: Heavy metals analysis of contaminated soil

Heavy Metal	Mean conc. before treatment (mg/kg)	Mean conc. after 1 week (mg/kg)	Mean conc. after 2 weeks (mg/kg)
Zinc	120.60	103.87	87.84
Chromium	22.67	59.37	13.64
Cadmium	11.23	27.93	6.05
Lead	18.43	41.33	17.83
Iron	187.27	171.85	100.63
Nickel	10.62	11.53	9.02

Concentrations of heavy metals were recorded before treatment of soil with a combination of calcium phosphate amendment and phosphate-solubilizing fungi. The metals analysed were zinc (Zn), chromium (Cr), cadmium (Cd), lead (Pb), iron (Fe) and nickel (Ni). Results showed Fe with the highest concentration at 187.27mg/kg followed by Zn 120.60mg/kg and Ni the lowest at 10.62mg/kg. Concentrations were measured after one week of treatment which indicated a significant drop in Zn, and Fe but an increase in the concentration of other metals. After the second week of treatment, there was a notable reduction in all the heavy metals analysed. The percentage reduction in heavy metal content of the soil is shown in Table 4. Fe

was reduced by 46.26%, closely followed by Cd (46.13%), while the least reduction was in Pb (7.60%)

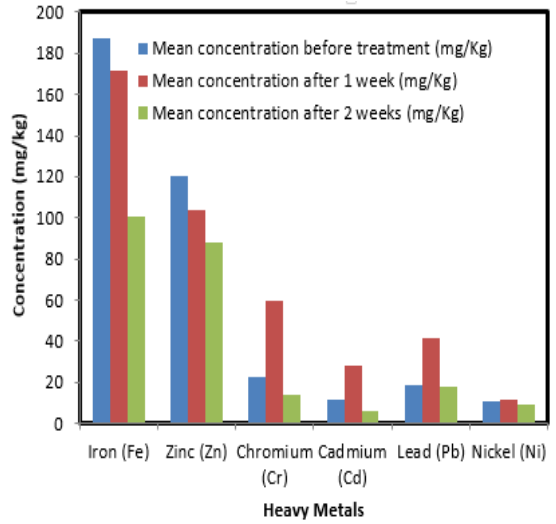

Fig 1: Heavy metal content of soil before and after treatment

Table 4: Percentage change in heavy metal concentration after treatment

Heavy metals	Percentage reduction in concentration (%)	
	Week 1	Week 2
Zinc (Zn)	**13.87**	**27.53**
Chromium (Cr)	**-161.89**	**39.83**
Cadmium (Cd)	**-148.71**	**46.13**
Lead (Pb)	**-124.25**	**7.60**
Iron (Fe)	**8.23**	**46.26**
Nickel (Ni)	**-85.57**	**15.07**

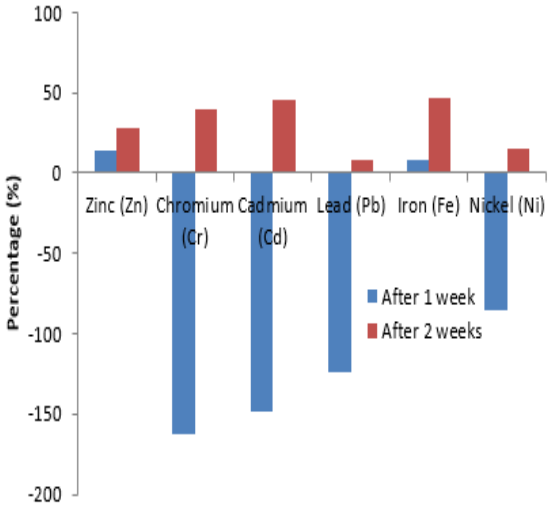

Fig 2: Percentage reduction in heavy metal concentration before and after treatment

This study found that by using calcium phosphate (hydroxyapatite) amendment produced from waste bones and isolated phosphate-solubilizing fungi (PSF), heavy metals in contaminated soil may be immobilized totally or to a modest degree. *Penicillium* species isolate utilized in this investigation were cultivated from fertile soil and selected for their abundance. Heavy metal analysis carried out on the polluted soil sample before treatment revealed high concentrations. In comparison with World Health Organization, permissible limits for heavy metals in soil, all parameters exceeded WHO limit indicating high rate of contamination which poses a threat to biodiversity. Iron (Fe) was observed to be the most abundant metal in the sample with 187.27 mg/kg making it below the maximum permissible limit. Iron is a commonly occurring metallic element in soil which range from 0.2 % to 55% (20,000 to 550,000 mg/kg) depending on soil type (USEPA, 2003). The concentration values obtained for zinc (Zn), was the second most abundant element in the sample with 120.60mg/kg greatly exceeded the WHO recommended range of 12-60mg/kg. The values observed for chromium 22.67 mg/kg were much higher than its limit (0.002-0.2 mg/kg), cadmium (Cd) 11.23 mg/kg exceeded WHO limits 0.002-0.5mg/kg, lead (Pb) at 18.43mg/kg surpassed the set limit 0.3-10 mg/kg and nickel (Ni) 10.62mg/kg exceeded its permissible limit 0.1-5mg/kg. Somewhat surprisingly, the first week of treatment lead to a reduction Zn by 13.87%, Fe by 8.23% but there was a notable spike in concentrations of Cr, Cd, Ni, and Pb. This may be as a result of PSF *Penicillium* sp. ability to decrease pH creating an acidic environment which enhances the solubility of phosphate in soil. This increment in concentration is attributed to the chelation of heavy metals from their compounds by the action of the hydroxyl and carboxyl groups of the organic acids produced by phosphate-solubilizing organisms, as reported by several studies (Sharma *et al.*, 2011; Pathak *et al.*, 2017). Li *et al.* (2016) agrees that compared to bacteria, PSF have ten times higher in their ability to secrete organic acids. The pH value in culture medium of PSF can be decreased to as low as 1 to 2. Therefore, PSF are considered a primary candidate in the pool of phosphate-solubilizing microorganisms (PSM). Generally, PSM could solubilize P minerals through various mechanisms. For example, microbial respiration and NH_4^+ ions can also enhance release of mobile inorganic P through releasing protons. After the second week, all metal concentrations were lower than they were at the beginning. Zn recorded 27.53% less than its initial concentration, Fe with 46.26% reduction, Cr with 39.83%, Cd second highest reduction at 46.13%, Ni decreased by 15.07% and Pb with the least reduction 7.60% demonstrating the significance of this study.

Conclusions: This research used a combination of calcium phosphate amendment and phosphate-

solubilizing fungus to immobilize heavy metals in polluted soil. Based on the results of the quantitative laboratory investigation, it can be determined that the immobilization effectiveness in contaminated soil was significant. Overall, the results indicate that a combination of a phosphate-solubilizing microorganism and calcium phosphate from bovine bones is effective in the immobilization of heavy metals in soil to reduce their bioavailability and is an effective treatment method. It is important to remember that this research lasted two weeks and the outcomes were as expected. Further study is needed, based on the conclusion, to better understand the environmental ramifications of these findings.

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