

**ASSESSMENT OF HYDROXYAPATITE AND PHOSPHATE SOLUBILISING
BACTERIA FOR HEAVY METAL REMEDIATION IN SOIL SAMPLES FROM A
MECHANIC WORKSHOP**



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BENIN CITY**

NOVEMBER, 2025

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**AN UNDERGRADUATE PROJECT WORK SUBMITTED TO THE DEPARTMENT OF
ENVIRONMENTAL MANAGEMENT AND TOXICOLOGY, FACULTY OF LIFE
SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE, NIGERIA; IN
PARTIALFULFILMENT OF THE REQUIREMENTS FOR AWARD OF BACHELOR
OF SCIENCE (B.Sc.) DEGREE IN ENVIRONMENTAL MANAGEMENT AND
TOXICOLOGY.**

NOVEMBER, 2025

CERTIFICATION

This is to certify that this research titled “**ASSESSMENT OF HYDROXYAPATITE AND PHOSPHATE SOLUBILISING BACTERIA FOR HEAVY METAL REMEDIATION IN SOIL SAMPLES FROM A MECHANIC WORKSHOP** ” was carried out by “**RUKEVWE RUTH EYETA (MISS)**” with matriculation number “**LSC2006925**” and presented to the Department of Environmental Management and Toxicology, Faculty of Life Sciences, University of Benin, Benin City; in partial fulfillment of the requirements for the award of Bachelor of Science (B.Sc.) in Environmental Management and Toxicology. It was conducted under suitable conditions, was carefully supervised and subsequently approved as having met the requirements for the award of Bachelor of Science degree in Environmental Management and Toxicology.

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Dr. O. Ogbeide
Project Supervisor

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Date

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Prof. (Mrs). E.T. Aisien
Head of Department

.....
Date

DECLARATION

I “**RUKEVWE RUTH EYETA**” declare that “**ASSESSMENT OF HYDROXYAPATITE AND PHOSPHATE SOLUBILISING BACTERIA FOR HEAVY METAL REMEDIATION IN SOIL SAMPLES FROM A MECHANIC WORKSHOP**” is my own work and that all sources that I have used or quoted have been acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other University.

RUKEVWE RUTH EYETA

.....
Date

DEDICATION

I would like to dedicate this project report to God Almighty for his love and undeserved kindness and also to all the individuals who have supported and guided me throughout this journey.

ACKNOWLEDGEMENTS

Foremost, I thank God for His grace, mercies and provision throughout my journey in school and for enabling me complete this program successfully.

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ABSTRACT

Heavy metal contamination from mechanic workshops poses a major environmental challenge due to the continuous accumulation of toxic metals in soil, which can affect microbial activities, reduce soil fertility, and pose health risks to humans and other organisms. This study assessed the effectiveness of hydroxyapatite (HAp) and phosphate solubilising bacteria (PSB) (*Pseudomonas sp.*) in remediating heavy metal contaminated soil collected from a mechanic workshop. The research involved four treatments: soil only (control), soil + PSB, soil + HAp, and soil + PSB + HAp. Microbial counts and heavy metal concentrations (Fe, Cu, and As) were analyzed after incubation. The total heterotrophic bacterial count (THBC) increased from 2.5×10^5 CFU/g in soil treated with PSB alone to $2.75 \pm 0.71 \times 10^5$ CFU/g in soil treated with both PSB and HAp, indicating enhanced microbial growth due to the combined amendment. The concentrations of iron (Fe) and copper (Cu) decreased significantly in all treated samples compared to the control. Fe reduced from 7561.97 mg/kg in the control to 6197.18 mg/kg in the combined treatment, while Cu decreased from 205.32 mg/kg to 150.19 mg/kg. Percentage reductions of 18.05% (Fe) and 26.85% (Cu) were recorded for the combined treatment, while arsenic (As) was not detected in any sample. These findings demonstrate that hydroxyapatite and *Pseudomonas sp.* act synergistically to immobilize and reduce heavy metal concentrations in contaminated soils. The combination improves microbial activity, enhances metal precipitation, and reduces the bioavailability of toxic metals. The study concludes that the combined use of hydroxyapatite and phosphate solubilising bacteria is an efficient, low-cost, and environmentally friendly method for remediating heavy metal-polluted soils from mechanic workshops in Nigeria.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Heavy metal contamination of soils is a serious threat to the global environment because of its high toxicity and nondegradability, particularly in developing countries like Nigeria, where there is poor management of waste and also intensive industrial and mechanical activities. Automobile and mechanic workshops are often contaminated by heavy metals like copper (Cu), iron (Fe), arsenic (As), zinc (Zn), etc. as a result of engine oil spills, battery and paint residues, lubricants, metal fragments, cleaning fluids and improper waste disposal (US EPA, 1997). These metals over time in the soil have the possibility of bioaccumulation and biomagnification in the food chain. They also pose risks to humans, animals, plants and ecosystems at large (Masindi and Muedi, 2018).

As a result of the negative impacts of heavy metals on the soil, different remediation approaches aimed at either reducing their bioavailability and mobility (immobilization) or enhancing their removal through stabilization or phytoextraction have been used. For example, the use of hydroxyapatite (HAp) which is a calcium phosphate mineral with the formula $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, has been proven to immobilize metals in contaminated soils via ion exchange, precipitation of metal phosphate compounds leading to increase in soil pH and improvement of soil structure (Katoh *et al*, 2017; Xu *et al*, 2021).

Another strategy includes the use of phosphate solubilizing bacteria (PSB) which are microorganisms that can increase the amount of phosphorus in soils by releasing organic acids that transform insoluble phosphate into soluble phosphate (PO_4^{3-}). PSB foster the precipitation

of heavy metals into less mobile forms, alter microbial activity and soil nutrient status (Hu *et al*, 2023; Peng *et al*, 2023)

However, despite the global body of laboratory proof on HAp and PSB separately, comparative studies assessing the effectiveness of hydroxyapatite derived specifically from cow bones and phosphate solubilizing bacteria on heavy metals in the Nigerian context remain limited

1.2 Statement of Problem

Mechanic workshops are often overlooked sources of soil pollution in Nigeria. Spilled motor oils and dismantled automotive parts release high levels of Pb, Cd, Cu, and Zn into the environment. Prolonged exposure to such metals poses health risks including neurotoxicity, kidney failure, and carcinogenesis in humans, and also disrupts microbial and plant activities in soils (Okoro *et al.*, 2020).

Although hydroxyapatite amendments and phosphate-solubilising microorganisms each show remediation promise, there is insufficient comparative, context-specific evidence to guide practitioners and regulators in Nigeria on which approach (or combination) is more effective at reducing total and bioavailable heavy-metal concentrations in mechanic workshop soils under local conditions. (Uyi *et al.*, 2023)

1.3 Aim and Objectives of the Study

The aim of this research is to evaluate the effect of hydroxyapatite amendment and phosphate-solubilizing bacteria inoculation on the heavy metal content of soil samples collected from a mechanic workshop. To achieve the stated aim, the specific objectives are:

- i. To determine baseline concentrations of selected heavy metals (Cu, As and Fe) in soil samples from the targeted mechanic workshop.

- ii. To evaluate the effect of hydroxyapatite amendment on the selected heavy metals after a defined incubation period.
- iii. To evaluate the effect of inoculation with locally isolated phosphate solubilizing bacteria on the heavy metals after the same incubation period.
- iv. To analyse the effectiveness of hydroxyapatite and PSB treatments in reducing heavy metal concentrations.
- v. To make practical recommendations for suitable remediation of mechanic workshop soils in Nigeria.

1.4 Significance of the Study

This study is significant in several ways. Environmentally, it addresses soil pollution from mechanic workshops which is a major but often ignored source of contamination in Nigerian cities. Scientifically, it contributes to knowledge on heavy-metal remediation by comparing mineral-based (hydroxyapatite) and biological (phosphate solubilising bacteria) approaches. The findings are also policy-relevant, providing evidence that can guide environmental agencies like NESREA and the Federal Ministry of Environment in formulating soil remediation strategies. In terms of public health, reducing heavy metal mobility helps prevent their entry into the food chain, protecting humans and animals from toxicity. Economically, the study promotes the use of low-cost, locally available materials like hydroxyapatite from cow bones and indigenous bacteria as affordable and sustainable options for soil remediation in Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview: Heavy Metal Concentration from Mechanic Workshops

Mechanic workshops are well-documented point sources of heavy metals (Pb, Cd, Cr, Ni, Cu, Zn, Fe) in urban soils due to spillage and disposal of used lubricants, worn brake linings, paints, batteries and metal filings. Soil near workshops often shows elevated total concentrations and increased bioavailable fractions compared with background soils, posing risks to workers and local residents via dust inhalation, dermal contact and food-chain transfer as noted by Diagi *et al.* (2023).

Mechanic-workshop contamination patterns are typically spatially heterogeneous (hotspots at drains, under work bays) and concentrated in surface horizons (0–15 cm) where human exposure is highest; microbial community structure and counts in contaminated soils are often affected by metal stress, but some metal-tolerant strains persist and may be exploitable for remediation (Balogun *et al.*, 2023).

2.2 Principles of Heavy Metal Remediation in Soils

Soil remediation approaches generally fall into removal (excavation, washing, phytoextraction) and stabilization/immobilization (in-situ chemical amendments that reduce mobility/bioavailability). Immobilization is often preferred for shallow, widespread contamination in urban settings because it is lower cost, less disruptive, and can be implemented in situ; amendments work by changing metal speciation, precipitating metals into low-solubility phases, or through adsorption to amendment surfaces. Hydroxyapatite (HAP) and biologically mediated phosphate release (e.g., via phosphate-solubilising bacteria, PSB) are two widely studied immobilization strategies (Brazdis *et al.*, 2021; Ahemad, 2015).

2.3 Hydroxyapatite (HAP): Nature, Synthesis from Animal Bone, and Remediation

Mechanisms

2.3.1 Chemistry and Adsorption/ Precipitation Mechanisms

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a calcium phosphate mineral chemically similar to bone mineral. It reduces heavy metal mobility through two principal mechanisms:

(1) surface adsorption / ion exchange where cationic metals substitute into surface sites of apatite; and

(2) surface-controlled precipitation where released phosphate reacts with dissolved metal ions (e.g., Pb^{2+} , Cd^{2+}) to form low-solubility metal phosphates (e.g., pyromorphite for lead), thereby lowering soluble/bioavailable metal fractions. The relative importance of adsorption compared precipitation depends on HAP particle size, surface area, soil pH and metal concentration.

(Brazdis *et al.*, 2021; Bigler *et al.*, 2024)

2.3.2 Synthesis of HAP from Animal Bone

Natural HAP can be produced from animal bones (bovine/cow, goat, fish) by deproteinization and calcination or by wet chemical and sol-gel processing. Using cow bone waste transforms an abundant agricultural/animal by-product into a low-cost remediation material particularly relevant in Nigeria where bone waste is locally available thus supporting circular economy principles. Natural bone-derived HAP often contains residual carbonates and trace elements that can enhance sorption behavior, though synthesis parameters (temperature, time, grinding) strongly influence crystal size, surface area and reactivity. Recent studies and local papers by Ojo *et al.* (2022) and Osuchukwu *et al.* (2022) have demonstrated extraction and characterization of bovine bone HAP for environmental or biomedical uses

2.3.3 Evidence for HAP Effectiveness in Soil Remediation

Brazdis *et al.* (2021), Yu *et al.* (2025) and Bigler *et al.*, (2024) conducted laboratory and field studies that proves that HAP effectively lowers extractable concentrations and plant uptake of Pb and Cd in contaminated soils. Formation of insoluble phosphate minerals (e.g., pyromorphite-like phases) is often reported as the long-term stabilization mechanism. Composite materials (e.g., carbon/HAP composites) and functionalized nano-HAP have been developed to increase surface area and adsorption capacity. However, performance depends on soil characteristics (pH, organic matter, competing cations) and the HAP to soil ratio; long-term stability and potential re-mobilization under changing environmental conditions warrant site-specific evaluation.

2.4 Phosphate Solubilising Bacteria (PSB): Mechanisms and Remediation Role

2.4.1 PSB: Functional Traits and Phosphate Release

Phosphate-solubilising bacteria are a functional group of bacteria able to release soluble phosphate from insoluble mineral or organic P sources (e.g., tricalcium phosphate, rock phosphate, apatite) mainly via secretion of organic acids (gluconic, citric, oxalic acids) and enzymes. In remediation contexts, PSB can increase the availability of phosphate in the rhizosphere or bulk soil, which can then react with metal cations to form insoluble metal-phosphate minerals, thus promoting immobilization. PSB can also stimulate plant growth (PGP, that is, plant growth-promoting traits) and improve phytostabilization/phytoextraction strategies (Ahemad, 2014; Yuan *et al.*, 2017).

2.4.2 PSB Tolerance to Metals and Direct Effects on Metals

Some PSB possess metal tolerance mechanisms (efflux pumps, sequestration, extracellular polymeric substances) and can be isolated from contaminated sites. Experimental work has shown that consortia of PSB can increase soil available phosphate and enhance immobilization

of Pb and Cd when used alongside phosphate amendments. However, the efficiency of PSB depends on the nature of the phosphate source, soil pH, and competition with native microbes; in highly acidic or extremely contaminated soils, PSB activity can be inhibited (Ahemad, 2014; Yuan *et al.*, 2017).

2.5 Combined Strategies: HAP + PSB, and other Amendment Combinations

Combining HAP (or other calcium phosphates) with PSB or organic supports can be synergistic: HAP provides a solid phosphate reservoir and sorptive surface while PSB can solubilize otherwise poorly available phosphate, sustaining the formation of stable metal phosphates in situ. Some studies report increased immobilization rates for Pb and Cd when microbial phosphate solubilization is coupled with added calcium phosphates (or HAP). Furthermore, composites like carbon/HAP can offer increased surface area; amendments such as KCl have been explored to accelerate transformations. Nonetheless, interactions are complex: excess soluble phosphate may transiently increase metal mobility before precipitation occurs, and microbial dynamics are sensitive to soil conditions all calling for controlled dosing and local pilot trials (Yu *et al.*, 2025; Bigler *et al.*, 2024; Yuan *et al.*, 2017)

2.6 Measurement of Remediation Success: Bioavailability and Ecotoxicological Endpoints

Successful immobilization is best assessed by changes in bioavailable fractions (e.g., DTPA-extractable metals, water-soluble fractions), sequential extraction (to track shifts to more recalcitrant fractions), reduced plant uptake in bioassays, and ecotoxicological tests (e.g., microbial respiration, earthworm tests). Total concentration declines are not expected for in-situ immobilization; rather, the key outcome is reduced mobility and bioavailability. Long-term monitoring is required to assess stability under varying pH and redox conditions (Brazdis *et al.*, 2021).

2.7 Nigerian Context: Soil Contamination in Mechanic Workshops and Local Remediation Studies

Alaekwe *et al.*,2018; Ale, 2025; Diagi *et al.*, 2023 and Balogun *et al.*, 2023 carried out multiple Nigerian studies and documented elevated heavy metal concentrations in soils from mechanic workshops across different states (e.g., Edo/Benin City, Imo/Nekede, Kogi/Anyigba, Zamfara/Gusau), consistently showing hotspots for several heavy metals and drawing attention to worker/public health risks (inhalation, ingestion, crop uptake). These studies provide both the local justification for studying HAP and PSB in Nigerian mechanic workshop soils and indicate soil types, contamination magnitudes and social contexts that affect remediation design. There is growing local research on producing HAP from animal bones in Nigerian and regional contexts (calcination and characterization), demonstrating feasibility of producing remediation materials locally from cow/bovine bones which is an approach that can reduce costs and align with local waste management. Field trials of HAP/PSB specifically in Nigerian mechanic workshop soils are more limited, indicating a knowledge gap and the need for site-specific studies that consider local soils, climate (tropical), and socio-technical constraints (Anayo *et al.*,2022; Abgozu and Wategire, 2025)

2.8 Factors Affecting Success and Limitations

Key factors that influence HAP/PSB remediation outcomes include:

- Soil pH: HAP performs better near neutral to slightly alkaline pH; extreme acidity can dissolve apatite or alter metal speciation. PSB effectiveness is also pH-dependent because microbial activity and organic acid dissociation change with pH (Brazdis *et al.*, 2021; Ahemad, 2014).

- Organic matter and competing cations: High organic matter and sorption sites can sequester metals and reduce direct HAP reactivity or compete for adsorption sites; complexation by humic substances may alter bioavailability (Brazdis *et al.*, 2021).
- HAP particle size and surface area: Nano or high-surface-area HAP and composites generally display higher removal capacity but raise cost and potential environmental safety questions (Yu *et al.*, 2025).
- Soil redox and long-term stability: Changing redox conditions (waterlogging) or acid inputs may remobilize previously stabilized metals; hence, durability and climate resilience must be considered (Brazdis *et al.*, 2021).

Limitations include potential short-term increases in soluble phosphate (which can cause eutrophication if leached), uncertain long-term stability in variable tropical conditions, and the need to ensure PSB strains are robust and non-pathogenic. Economic feasibility and local production quality control (for bone-derived HAP) are also practical considerations.

2.9 Literature Review Analysis Table

No.	Citation (Author, Year)	Location / sample type	Contaminants measured	Remediation material / approach	Main result / relevance
1	Balogun <i>et al.</i> , 2023 (Assessment of soils in Benin mechanic workshops).	Benin City, Edo State, Nigeria — mechanic workshop soils	Pb, Cd, Zn, others; microbial counts	Baseline contamination assessment	Showed elevated heavy metals and altered microbial counts — local justification for remediation study.
2	Local study, Nekede/Orji,	Mechanic workshop soils	Pb, Cd, Zn, Cu	Contamination	Confirmed surface enrichment and spatial

	Imo State (2023).	(Imo State)		assessment	heterogeneity — typical patterns relevant to sampling design.
3	MDPI review — hydroxyapatite for heavy metals (Brazdis <i>et al.</i> , 2021).	Global literature review	Pb, Cd, Cr, Cu, Zn	Hydroxyapatite adsorbents	HAP reduces bioavailability via adsorption and phosphate precipitation; outlines factors controlling effectiveness.
4	Ahemad and Khan, 2014 (PSB review).	Review	Pb, Cd, other metals (in phytoremediation contexts)	PSB-assisted phytoremediation	Mechanisms of PSB action (organic acids, enzymes); role in improving phytoremediation/immobilization.
5	Study: HAP from bovine bone (Agbozu <i>et al.</i> , 2025) — extraction and characterization.	Laboratory extraction from bovine/cow bone	—	Bone-derived HAP produced & characterized	Demonstrated feasibility of producing HAP from bovine bone (protocols, properties) — supports local sourcing.
6	Carbon/HAP composite study (MDPI Water, 2025) — CHAP for metal removal.	Laboratory (animal bone derived CHAP)	Pb, Cd etc.	Carbon/HAP composites	Enhanced removal vs bare HAP; suggests composite options for higher capacity.
7	Application of PSB to immobilize Pb	Experimental soils	Pb, Cd	PSB consortia + calcium	PSB increased available P and immobilization rates for Pb and Cd

	and Cd (research article).			phosphate	when Ca-phosphate was present.
8	Regional study: Gusau mechanic workshops (Gusau).	Gusau, Nigeria mechanic soils	Cr, Pb, others	Contamination assessment	Confirms mechanic workshops across Nigeria as heavy metal hotspots.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Benin City, Edo State, in Nigeria. The area lies between latitudes $6^{\circ}23'18''$ N and $6^{\circ}23'47''$ N, and longitudes $5^{\circ}37'3''$ E and $5^{\circ}37'19''$ E. It is located in the tropical rainforest belt of southern Nigeria.

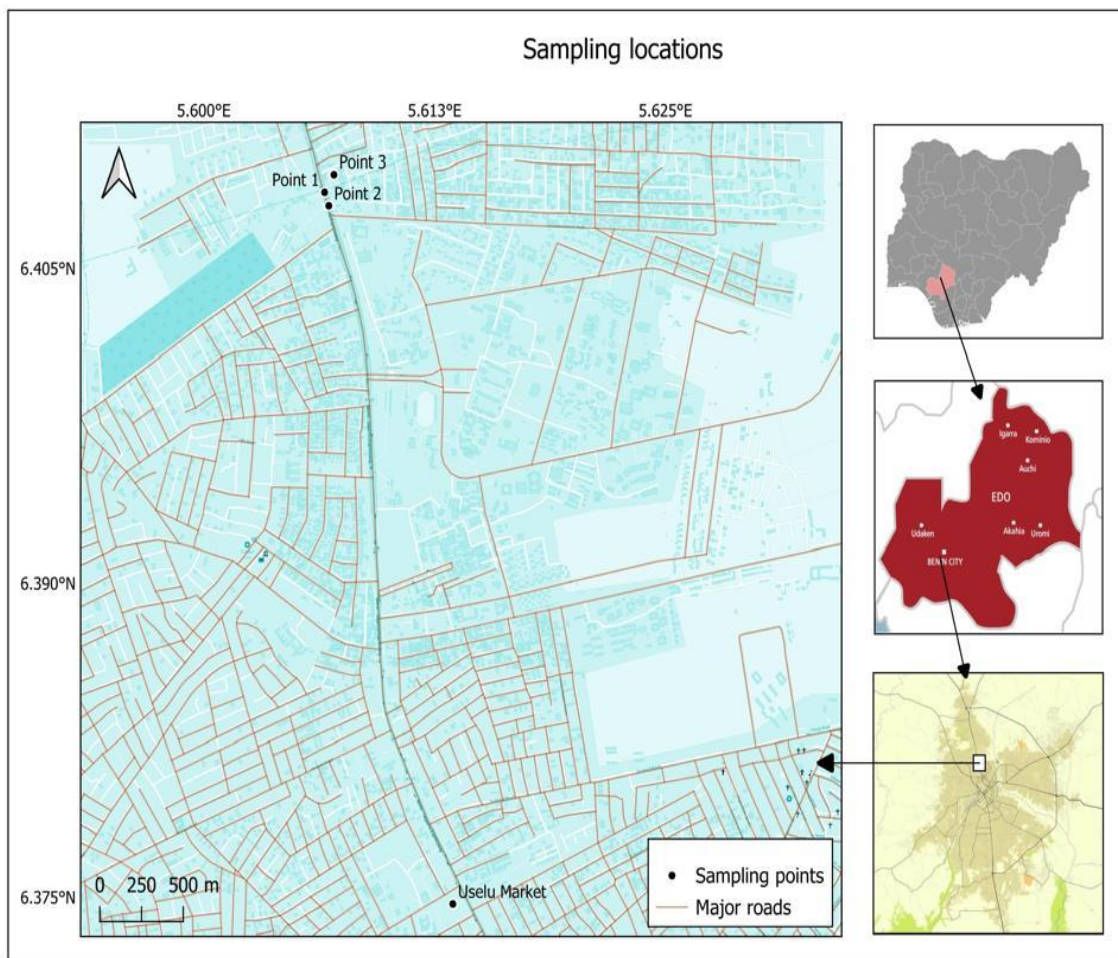


Figure 3.1: Map of the study area showing sampling location

3.2 Sample Collection and Preparation

3.2.1 Soil Sample Collection

Soil samples were collected from a mechanic workshop located in Ekosodin, Benin City, Edo State, Nigeria, which has been operational for some years. The site was selected based on visible signs of long-term petroleum contamination, including oil-stained soils, poor vegetation cover, and hydrocarbon odour. Sampling was done at three different points within the workshop area using a sterile spatula at a depth of 10-20cm. The samples from all points were pooled together to form a composite sample, thoroughly mixed, and placed in sterile, airtight polyethylene bags to prevent volatilization of hydrocarbons.

Rhizosphere samples were collected from fertile land in the University of Benin by gently uprooting healthy plants and brushing off the soil adhering to the root surfaces into sterile containers. Control (uncontaminated) soil samples were collected from an undisturbed vegetative area (approximately 2 km away from the mechanic workshop). The samples were labeled appropriately, transported to the laboratory in ice-cooled containers, and stored at 4°C prior to analysis and experimental setup.

3.2.2 Soil Pre-Treatment

In the laboratory, the collected soil samples were air-dried at room temperature (25–35°C) for 7 days to reduce moisture content and facilitate uniform mixing. The dried soils were then sieved through a 2.8 mm (7 British Standard Sieve) mesh to remove stones, plant debris, and other coarse materials. The homogenized soil sample from the mechanic workshop served as the experimental soil, while the uncontaminated soil was used as the negative control.



PLATE 3.2.2: Soil sample being sieved

3.2.3 Storage and Handling

After pre-treatment, soils were stored in clean plastic containers at ambient temperature (25–30°C) and protected from direct sunlight to prevent further degradation of hydrocarbons before experimental use. All glassware and tools used during handling were sterilized to minimize microbial interference

3.3 Preparation of Hydroxyapatite

3.3.1 Collection and Cleaning of Bones

Old cow bones (bovine) were sourced from a local abattoir in Uselu market, Benin City, Edo state. Bovine bones were washed with water and 70% ethanol to remove impurities and residual organic matter.

3.3.2 Calcination and Pulverization

The cleaned bones were sun-dried for 48 hours and oven-dried at 105°C for 24 hours. The dried bovine bones were crushed into smaller pieces with a mallet and pulverized to powder. For the extraction of calcium phosphate from the bones, thermal decomposition was used. Pulverized bones were placed in a ceramic crucible and heated in a muffle furnace at 800 °C for 3 hours with heating rate of 10°C/min to eliminate organic constituents in the bovine bone to achieve crystalline hydroxyapatite (Agbozu and Wategire, 2025).

3.4 Isolation and Characterization of Phosphate-Solubilizing Bacteria (PSB)

3.4.1 Preparation of Soil Suspension

In 250 mL Erlenmeyer flasks, 10 grams of each soil sample were suspended in 90 mL of sterile distilled water. To separate microbial cells from soil particles, the suspension was shaken for 30 minutes at 150 rpm on a rotary shaker. Next, sterile distilled water was used to prepare serial dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} .

3.4.2 Isolation of Bacteria using Pour Plate

One millilitre portions of each dilution were aseptically moved into sterile Petri dishes. Each dish received 15–20 mL of molten nutrient agar that had been cooled to 45 °C. To guarantee that the inoculum was distributed evenly, the plates were gently swirled before being left to harden. The incubation period was 24 to 48 hours at 28 ± 2 °C. To obtain pure isolates, morphologically distinct colonies were chosen and sub cultured onto fresh nutrient agar plates (Ihoeghian *et al.*, 2023; Isagba *et al.*, 2023).

3.4.3 Screening for Phosphate Solubilization

Pikovskaya's agar medium, which has tricalcium phosphate (TCP) as the only insoluble phosphorus source, was used to screen purified bacterial isolates for phosphate-solubilizing ability. A loopful of the isolate was applied to the agar surface in order to accomplish spot inoculation. For three to seven days, the plates were incubated at 28 ± 2 °C. The clear halo zones that formed around the colonies as a result of TCP disintegration were indicative of phosphate solubilisation (Ihoeghian *et al.*, 2023; Isagba *et al.*, 2023).

3.4.4 Measurement of Solubilization Index (SI)

A digital calliper was used to measure the diameter of the bacterial colony and the diameter of the clear zone, which included the colony, following a three-day incubation period. The following formula was used to compute the solubilisation index:

$$SI = \frac{\text{Halo zone diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

Higher SI isolates were chosen for additional characterisation since they were thought to be more effective phosphate solubilizers.

3.4.5 Characterization of PSB Isolates

To ascertain their phenotypic characteristics, a few phosphate-solubilizing isolates were put through Gram staining and simple biochemical assays like catalase and oxidase activity. The bacterial isolates were initially identified and categorised using these assays. Pure isolates were characterized using standard morphological and biochemical tests as described by Holt *et al.* (1994) and APHA (2017). Tests performed included:

- **Gram staining:** Each bacterial isolate was prepared as a thin smear on a clean glass slide, heat-fixed, and then sequentially stained with crystal violet (primary stain) for three minutes, rinsed with water, and then stained with Gram's iodine (mordant) for three minutes, followed by another round of rinsing. The decolorisation step involved using 95% ethanol (decolorizer) for ten to fifteen seconds, counterstained, and safranin for one to three minutes. After air drying, the slides were inspected using an oil immersion light microscope at 1000× magnification. Based on the structural variations in their cell walls, bacteria can be distinguished via gram staining. Under a microscope, gram-positive bacteria appear purple because of their thick peptidoglycan coating, which helps them preserve the crystal violet-iodine combination. Gram- negative bacteria appear pink or red because they have a weaker peptidoglycan layer and an outer membrane that allows the dye to be washed off and replaced by the counterstain (safranin). Gram-positive bacteria do not lose the primary stain's colour.
- **KOH String Test:** On a sterile glass slide, a drop of 3% KOH solution was combined with a loopful of fresh bacterial culture. After 30 seconds of stirring, the fluid was gently raised with the loop to see if strings formed. One quick way to verify the Gram reaction is the KOH string test. In 3% potassium hydroxide, gram-negative bacteria lyse,

releasing DNA that, when raised with a loop, creates a thick string. Gram-positive bacteria do not make a string and are resistant to lysis. Gram-negative bacteria are therefore indicated by a positive string test result and vice versa.

- **Catalase Test:** Using a sterile loop, a colony of the test organism was put to a clean slide containing a drop of 3% hydrogen peroxide. A favourable response is shown by the cells in the loop bubbling right away. The catalase test determines whether the catalase enzyme, which converts hydrogen peroxide into oxygen and water, is present. A positive catalase result is indicated by the visible bubbling that result from the release of oxygen, whereas a negative catalase result is indicated by the absence of gas bubbles or bubbling.
- **Citrate Utilization Test:** The manufacturer's instructions were followed to make Simon's citrate agar in slant test tube bottles. After inoculating the Simmons citrate agar slants with the test organisms, they were incubated for 24 to 48 hours at 37°C. A record of the colour shift was made. This test assesses the capacity of bacteria to use citrate as their only carbon source. When citrate is used, alkaline byproducts are produced, which cause the bromothymol blue indicator to change from green to blue. A bad outcome is represented by no colour change (green), whereas a favourable outcome is shown by blue colouration.
- **Triple Sugar Iron (TSI) Agar Test:** Stabbing the butt and streaking the slant's surface allowed for the preparation and inoculation of TSI agar slants. The tubes were incubated for a full day at 37°C. Black precipitate, gas production, and slant/butt colour were among the observations. TSI agar distinguishes between bacteria according to their capacity to produce hydrogen sulphide (H₂S) and gas, as well as their ability to ferment glucose, lactose, and sucrose. Sugar fermentation is shown by colour changes in the slant

and butt, while H₂S generation is indicated by black precipitate. Below is a reading and analysis of the results:

- a. Yellow butt/slant: Fermentation of sugars
- b. Red slant/yellow butt: Glucose fermentation only
- c. Black precipitate: H₂S production
- d. Gas bubbles/cracks: Gas production
- e. Red butt/slant: No fermentation

Molecular identification was done via 16S rRNA gene sequencing, and the isolate was confirmed as a strain of *Pseudomonas sp.*, consistent with earlier reports of phosphate-solubilising *Pseudomonads* from contaminated Nigerian soils (Eze *et al.*, 2022).

3.4.6 Preservation of Bacterial Isolates

The bacterial isolates were kept on nutrient agar slants at 4°C to guarantee long-term availability. Furthermore, 20% v/v glycerol stocks were made and kept at -80°C for later use (Holt *et al.*, 1994; APHA, 2017)



PLATE 3.4.6: Hydroxyapatite and *Pseudomonas* sp.

3.5 Experimental Design for Study

3.5.1 Overview of Experimental Setup

The experiment was designed to evaluate the individual and synergistic effects of phosphate-solubilising bacteria (*Pseudomonas sp.*) and hydroxyapatite (HAp) on the remediation of petroleum-contaminated soil collected from a mechanic workshop. The experiment followed a completely randomized design (CRD) with four treatments

3.5.2 Experimental Treatments

Table 3.5.2: Experimental setup for treatment

The experimental setup consisted of the following treatments:

Treatment Code	Description
T ₁	Contaminated soil only
T ₂	Contaminated soil + Hydroxyapatite (HAp) only
T ₃	Contaminated soil + <i>Pseudomonas sp.</i> only
T ₄	Contaminated soil + HAp + <i>Pseudomonas sp.</i>

3.5.3 Experimental Procedure

- 900 g of air-dried contaminated soil was weighed into plastic pots (4L capacity).
- 40g of HAp were mixed thoroughly with the soil.
- *Pseudomonas sp.* inoculum (10 mL) was added to designated treatments.
- Moisture content was adjusted to 60% of water-holding capacity and maintained throughout the 14 day incubation period under controlled laboratory conditions (temperature $28 \pm 2^\circ\text{C}$) to simulate tropical soil environments typical of southern Nigeria.
- The soils were gently mixed at daily intervals to maintain aeration.



● **PLATE 3.5.3.1: Addition of hydroxyapatite to soil samples**



PLATE3.5.3.2: Treatment setup for soil samples

3.6 Determination of Heavy Metal Content

3.6.1 Digestion Procedure

About 1 g of the finely ground sample was weighed into a conical flask, after which 10 ml of a nitric–perchloric acid mixture was added and allowed to soak overnight. A small glass funnel was then inserted to serve as a reflux condenser, and the mixture was heated for 1 hour at 150 °C. The temperature was gradually increased to 235 °C, and heating continued until dense white fumes appeared and a colourless solution was obtained. The digest was then poured into a 100 ml volumetric flask, rinsed five times with distilled water, and the washings were added to the flask before making up to the mark with distilled water. Blank samples were prepared following the same procedure but without any sample. The resulting filtrates were subsequently analysed for heavy metals using Atomic Absorption Spectrophotometry (AAS).

3.6.2 Calibration and Analysis

Single-element standards were prepared by diluting 1000 mg/L stock solutions of the individual elements (Fe, Cu and As). A minimum of five working standard solutions were prepared daily from the stock solutions, with concentrations ranging between 0.1 mg/L and 10 mg/L. External calibration was performed using deionised water and a set of calibration standards for each element, after which calibration curves were generated for the respective metals. The digested samples and blanks were then analysed using Atomic Absorption Spectrophotometry (AAS) to obtain their absorbance values. The concentrations of the metals in the samples were subsequently calculated using the equations derived from the calibration curves.

3.6.3 Quality Assurance

First, duplicate blanks are aspirated from acidified deionised water. As quality control samples, duplicates and lab control samples are used.

CHAPTER FOUR

RESULTS

4.1 Microbial Count

Table 4.1 shows the total heterotrophic bacterial count (THBC) of the samples. It was determined to assess the effect of phosphate solubilising bacteria (*Pseudomonas sp.*) and hydroxyapatite (HAp) on microbial proliferation within the treated soils. The microbial count increased in all treated soils, with the highest in Soil + PSB + HAp (2.75×10^5 CFU/g). This the soil inoculated with *Pseudomonas* and HAp compared to the soil treated with *Pseudomonas* alone. The increase in the microbial population in the soil amended with both HAp and PSB suggests a synergistic interaction that favours microbial growth. The presence of hydroxyapatite likely improved soil physicochemical conditions and provided an environment conducive for bacterial proliferation. This implies that the combination of HAp and PSB enhances microbial activity, which is beneficial for metal immobilization and nutrient transformation processes in contaminated soils.

Table 4.1: Total heterotrophic bacterial count

	THBC ($\times 10^5$ CFU/g)
Soil + PSB	2.50 ± 0.00
Soil + PSB + HAP	2.75 ± 0.71

4.2 Concentration of Heavy Metals

Table 4.2 and figure 4.2 display the concentrations of iron (Fe), copper (Cu) and arsenic (As). The results show that no As was detected in the samples analysed which suggests that it was absent or present in trace quantities below the detection limit of the analytical method used which further suggests the contamination source was primarily from ferrous and copper based mechanical materials rather than arsenic containing compounds. The initial high Fe concentration (7561.97 mg/kg) in untreated soil reflects severe contamination associated with mechanical and metalworking activities in the study area. The soil amended with both hydroxyapatite and *Pseudomonas* sp. recorded the lowest concentrations of Fe (6197.18 mg/kg) and Cu (150.19 mg/kg), indicating a higher level of remediation. The reduction pattern followed the trend:

Soil only > Soil + PSB > Soil + HAp > Soil + PSB + HAp

This indicates that hydroxyapatite alone was more effective than PSB alone in immobilizing metals, but the combination of both provided the best remediation effect.

Table 4.2: Concentration of heavy metals in samples

Treatment	Iron (Fe) (mg/kg)	Copper (Cu) (mg/kg)	Arsenic (As)
Soil only	7561.97	205.32	Not detected
Soil + PSB	7007.04	198.47	Not detected
Soil + HAp	6901.41	174.32	Not detected
Soil + PSB + HAp	6197.18	150.19	Not detected

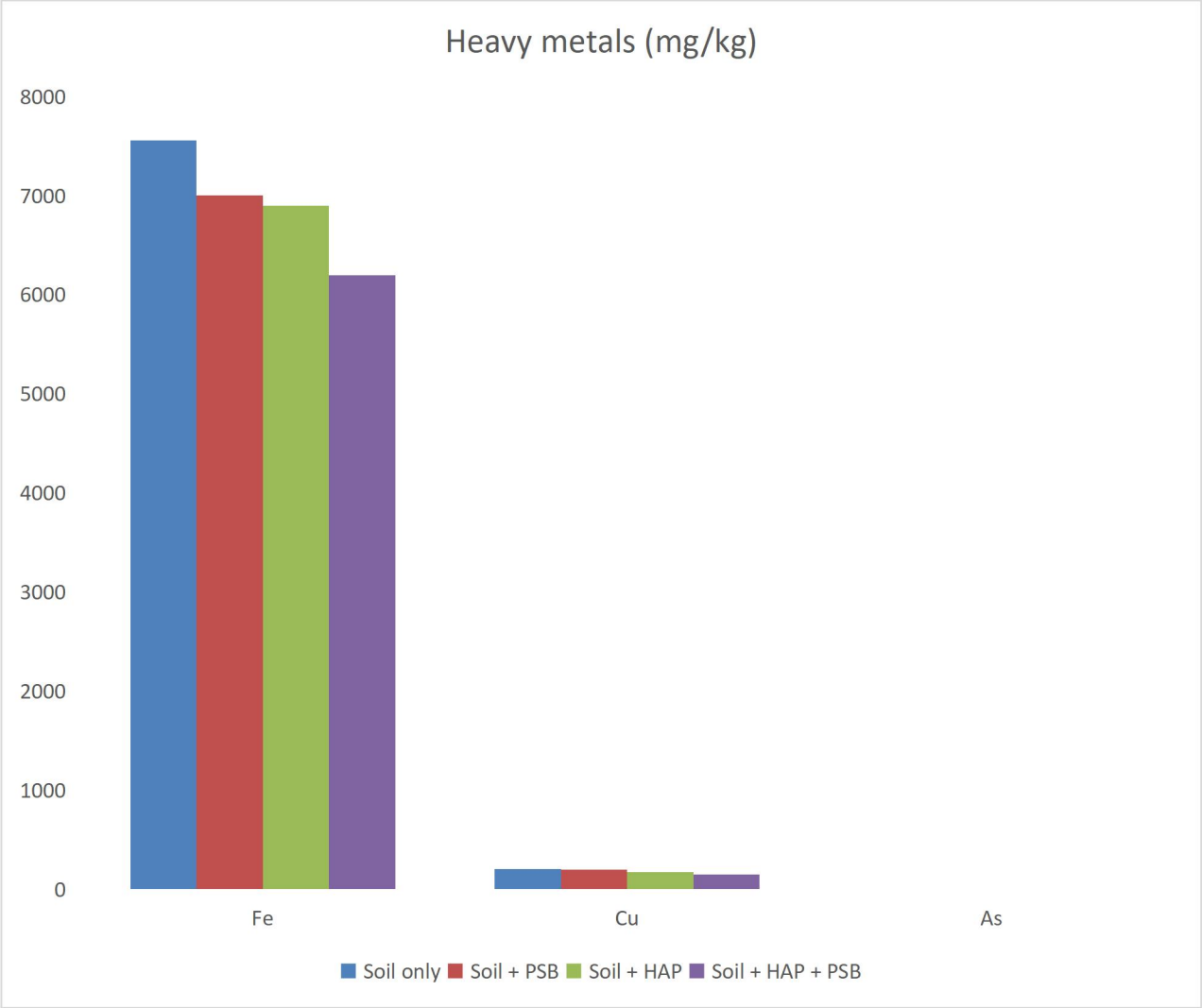


Figure 4.2: Concentration of heavy metals in samples

4.3 Reduction in Heavy Metal Concentrations

Table 4.3 shows the reduction in percentage in bioavailable metal concentrations after 14 days of treatment. It was calculated to evaluate the efficiency of each treatment. The data indicate that the combined use of hydroxyapatite and *Pseudomonas sp.* resulted in the highest reduction in both Fe and Cu concentrations, with percentage reductions of 18.05% and 26.85%, respectively while arsenic remained undetected in all treatments. The higher reduction in the combined treatment could be attributed to the complementary mechanisms of both agents:

- I. Hydroxyapatite acts through ion exchange and precipitation reactions that immobilize metals into stable phosphate complexes.
- II. *Pseudomonas sp.* enhances bioavailability and facilitates the transformation of metals into less toxic or immobile forms through biosorption and biomineralization.

The results demonstrate that coupling biological (PSB) and mineral (HAp) amendments significantly improves heavy metal remediation efficiency in contaminated soils, making it a promising eco-friendly approach for reclaiming polluted mechanic workshop soils.

Table 4.3: Percentage reduction of heavy metals

	% removal		
	Fe	Cu	As
Soil + PSB	7.338432	3.336256	ND
Soil + HAP	8.73529	15.09838	ND
Soil + HAP + PSB	18.04807	26.85077	ND

CHAPTER FIVE

DISCUSSION

5.1 Discussion

The findings of this study clearly showed that the combined use of hydroxyapatite (HAp) derived from natural sources and phosphate solubilising bacteria (*Pseudomonas sp*) significantly reduced the concentrations of heavy metals in contaminated soil collected from a mechanic workshop in Benin City. The results demonstrated the effectiveness of both biological and mineral-based remediation strategies, particularly when applied together.

In treated soils, the microbial count increased, particularly in the sample that included both PSB and HAp (2.75×10^5 CFU/g), suggesting enhanced microbial activity compared to soil with Psb alone (2.5×10^5 CFU/g). This implies that the combined treatment created a more favorable environment for the growth of *Pseudomonas sp*. The reduction in metal toxicity and improved nutrient availability likely contributed to enhanced bacterial proliferation. Hydroxyapatite acts as a buffering agent that stabilizes soil pH and reduces the bioavailability of toxic metals, while PSB release organic acids that solubilize phosphate and promote microbial activity (Hu *et al.*, 2023).

The concentrations of iron (Fe) and copper (Cu) were significantly reduced in all treated soils compared with the untreated control. The highest reduction was recorded in the soil treated with both PSB and HAp treatment where Fe decreased from 7561.97 mg/kg to 6197.18 mg/kg and Cu from 205.32 mg/kg to 150.19 mg/kg. This corresponds to percentage reductions of 18.05% and 26.85%, respectively. This significant reduction demonstrates the synergistic effect of combining both treatments enhances the immobilization and bio-precipitation of heavy metals.

The mechanisms behind this reduction can be explained by the complementary functions of HAp and PSB. Hydroxyapatite has a high affinity for metal ions, allowing it to immobilize heavy metals through adsorption, ion exchange, and precipitation processes. On the other hand, *Pseudomonas sp.*, a phosphate solubilising bacterium, can secrete organic acids that mobilize phosphate ions from HAp. These phosphate ions subsequently react with heavy metal cations to form insoluble metal phosphates, which are stable and non-bioavailable. Additionally, the metabolic activities of *Pseudomonas sp.* can alter soil pH, promote biosorption, and facilitate the reduction of metal toxicity (Hu *et al.*, 2023; Peng *et al.*, 2023).

The absence of arsenic (As) in all samples implies that arsenic either occurs at negligible levels in the study area or is not a dominant contaminant and indicates that the contamination in the studied mechanic workshop mostly stemmed from ferrous and copper-based compounds characteristic of automotive waste such as engine parts, lubricants, and metal scrap (Ajeh *et al.*, 2022).

In general, using PSB and HAp together increased the biological and chemical qualities of the soil. This made it a cheap and eco-friendly way to clean up heavy metals in Nigeria. Using cow bones from the area also helps with sustainable waste management, which is in line with what Agbozu and Wategire (2025) found when they looked at the possibilities of HAp made from bones for cleaning up the environment.

5.2 Conclusion

The study showed that phosphate-solubilizing bacteria (*Pseudomonas sp.*) and hydroxyapatite made from cow bones are both efficient, affordable, and environmentally beneficial solutions for cleaning up heavy-metal-contaminated soils, particularly those connected to auto repair operations. Heavy metals were mainly immobilised by hydroxyapatite by ion exchange and

adsorption, and soil recovery was aided by phosphate-solubilizing bacteria that increased metal precipitation and nutrient bioavailability.

Nonetheless, the combined treatment gave the highest remediation efficiency and turned out to be the most effective, suggesting that an integrated physicochemical and biological remediation strategy performs better than either technique applied separately. The increased microbial population observed in the HAp + PSB treatment indicates a positive interaction between both agents, leading to enhanced microbial activity and improved soil health. The reduction of Fe and Cu levels, and the absence of As, further demonstrate the remediation potential of the treatment. This discovery provides a useful model for comparable contaminated settings throughout Nigeria and similar developing regions and is consistent with international efforts towards sustainable soil restoration using locally accessible materials and native microbial resources.

5.3 Recommendations

The study's findings have several practical implications. The combined use of hydroxyapatite and phosphate solubilising bacteria should be tested on a larger scale in contaminated areas for example, industrial zones to assess its real world effectiveness. Long-term monitoring is necessary to ensure that immobilized metals remain stable under varying environmental conditions.

Awareness programs by environmental agencies and NGOs should educate mechanics and workshop owners on proper waste management and the benefits of eco-friendly remediation methods. Government bodies like NESREA should incorporate HAp and PSB-based bioremediation into national pollution control policies and offer incentives for sustainable practices. Collaboration between research institutions and industries is also vital to improve hydroxyapatite production and identify effective PSB strains for different soil types. Finally,

public education should emphasize the health risks of heavy metal pollution and the importance of soil remediation in ensuring environmental and food safety.

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