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Remediation of Leachate-Metal-Contaminated Soil Using Selected Bacterial Consortia

Chijioke Emenike [1,2,*], Patricia Omo-Okoro [1], Agamuthu Pariatamby [3], Jayanthi Barasarathi [4] and Fauziah Shahul Hamid [5]

[1] Department of Plant, Food, and Environmental Sciences, Dalhousie University, Truro, NS B2N 5E3, Canada; patricia.omo-okoro@dal.ca
[2] Natural and Applied Sciences, Hezekiah University, Nkwerre 471119, Nigeria
[3] Jeffrey Sachs Center on Sustainable Development, Sunway University, Bandar Sunway 47500, Malaysia; agamutup@sunway.edu.my
[4] Faculty of Health & Life Sciences, Inti International University, Nilai 71800, Malaysia; jayanthi.baras@newinti.edu.my
[5] Institute of Biological Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia; fauziahsh@um.edu.my
* Correspondence: chijioke.emenike@dal.ca

Abstract: Approximately 95% of urban solid waste worldwide is disposed of in landfills. About 14 million metric tonnes of this municipal solid waste are disposed of in landfills every year in Malaysia, illustrating the importance of landfills. Landfill leachate is a liquid that is generated when precipitation percolates through waste disposed of in a landfill. High concentrations of heavy metal(loid)s, organic matter that has been dissolved and/or suspended, and inorganic substances, including phosphorus, ammonium, and sulphate, are present in landfill leachate. Globally, there is an urgent need for efficient remediation strategies for leachate-metal-contaminated soils. The present study expatiates on the physicochemical conditions and heavy metal(loid)s' concentrations present in leachate samples obtained from four landfills in Malaysia, namely, Air Hitam Sanitary Landfill, Jeram Sanitary landfill, Bukit Beruntung landfill, and Taman Beringin Landfill, and explores bioaugmentation for the remediation of leachate-metal-contaminated soil. Leachate samples (replicates) were taken from all four landfills. Heavy metal(loids) in the collected leachate samples were quantified using inductively coupled plasma mass spectrometry. The microbial strains used for bioaugmentation were isolated from the soil sample collected from Taman Beringin Landfill. X-ray fluorescence spectrometry was used to analyze heavy metal(loid)s in the soil, prior to the isolation of microbes. The results of the present study show that the treatments inoculated with the isolated bacteria had greater potential for bioremediation than the control experiment. Of the nine isolated microbial strains, the treatment regimen involving only three strains (all Gram-positive bacteria) exhibited the highest removal efficiency for heavy metal(loid)s, as observed from most of the results. With regard to new findings, a significant outcome from the present study is that selectively blended microbial species are more effective in the remediation of leachate-metal-contaminated soil, in comparison to a treatment containing a higher number of microbial species and therefore increased diversity. Although the leachate and soil samples were collected from Malaysia, there is a global appeal for the bioremediation strategy applied in this study.

Keywords: bacteria consortia; bioremediation; bioaugmentation; toxic metals; landfill sites; leachate

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1. Introduction

Urban solid waste management places a heavy strain on society and exacerbates economic and environmental issues. Such issues include climate change impacts, methane emission, land degradation, and water and air pollution. Consequently, municipal solid waste (MSW) landfills are designed and located to reduce these environmental and economic issues. Approximately 95% of urban solid waste worldwide is disposed of in

landfills [1]. Landfills are commonly used worldwide to minimize waste accumulation, with both biodegradable and non-biodegradable waste being deposited in landfills [2]. However, landfills pose several risks as waste may release harmful elements into the environment [1]. Leachate is a liquid substance that results from the decomposition of waste materials in landfills [3]. It is highly concentrated and contains a mixture of hazardous chemicals. This harmful substance constitutes a major threat to the environment, particularly to soil and groundwater [3]. Typically, leachate is generated by surface runoff, precipitation, and infiltration water that seeps through waste and during the biodegradation of biodegradable waste. It is a key secondary pollutant emanating from conventional waste treatment [4]. Leachate pollutes the soil as it flows through it and simultaneously poses a threat to vegetation along its course. In unsaturated soil, this migration typically occurs vertically; however, in saturated soil or above an impermeable layer of soil, leachate migrates horizontally [5].

A variety of components such as phenols, aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), sulphate, phosphorus, ammonium, fluvic and humic acids (frequently present as chemical oxygen demand (COD)), and toxic metals make up the majority of landfill leachate [6,7]. Leachate-metal-contaminated soil can cause detrimental effects on both the ecosystem and human health [8,9]. Microbial remediation is utilized in the present study as a technique for the treatment of leachate-metal-contaminated soils. Microbial remediation offers a sustainable and cost-effective approach to mitigate the adverse impacts of heavy metal(loid)s contamination [10].

The research reported is a two-phase study. Firstly, the authors present the findings from the physicochemical assessments of leachate samples collected individually (replications) from four different landfills in Malaysia. Two sanitary landfills, Air Hitam Sanitary Landfill (AHL) and Jeram Sanitary Landfill (JSL), and two non-sanitary landfills, Bukit Beruntung Landfill (BBL) and Taman Beringin Landfill (TBL), were investigated. Both AHL and TBL are closed while JSL and BBL are active. Secondly, the study further explores the effectiveness of using microbial strains that were isolated from the soil taken from TBL to remediate leachate-metal-contaminated soil via bioremediation experiments (involving four microcosms). The 'non-contaminated urban soil' used to set up the microcosms was obtained from a garden inside the University of Malaya in Malaysia. Furthermore, the leachate that was used to artificially contaminate the 'non-contaminated soil' was collected from JSL. The soil from TBL was selected owing to TBL's direct contact with soil cores as it is non-sanitary (no liners). The leachate used for artificial contamination was taken from JSL because the highest concentrations for metal and metalloids in leachate samples were recorded for JSL. Both the soil obtained from TBL and the leachate derived from JSL were characterized. With regard to the garden soil, if any bioremediation-facilitating bacteria species are present in the soil, then this could cause natural attenuation. This further forms a basis for its use as a 'control'. This study aims to understand the possible distribution of bacteria in soil contaminated with leachate; assess the potential effects of bioaugmenting soil laden with heavy metal(loid)s via leachate contamination; and evaluate the removal rates of the heavy metal(loid)s in the treated soil using a kinetic model. The four landfills were adopted as variables that potentially influence the level of leachate toxicity in Malaysia.

This study presents multiple results emanating from the physicochemical assessments of four different landfills in Malaysia. A broad audience including researchers, environmental scientists, policy makers, and government could find the results invaluable. The present work elucidates the possibility of obtaining enhanced bioremediation by employing a treatment with a smaller number of microbial species, as against a treatment involving a higher number of microbes. Consequently, the research reported lends credence to a growing idea in microbial remediation. This emerging perspective suggests that microbial manipulation may outperform increased diversity. Findings from the present work will add to the existing knowledge bordering on the understanding of the microbial remediation of

leachate-metal-contaminated soils. Collectively, these factors constitute the significance of the present study.

2. Materials and Method

Procedures and protocols such as the sample collection, identification, and quantification of metals, isolation of microbes, experimental setups, and bioaugmentation were employed in this study. Some leachate samples were collected from pipes connected to the landfill cells of AHL and JSL, while the rest were obtained from overflows and seepages adjoining BBL and TBL. Leachate samples (replicates) were collected individually from each of the 4 different landfills. Some of the international guidelines that were applied during the analyses of the chemical constituents of the leachate samples include the Environmental Protection Agency [11], the Association of Official Analytical Chemists [12], and the American Public Health Association [13].

2.1. Soil and Leachate Sample Collection

Soil samples were divided into two classes: contaminated and uncontaminated soil. The uncontaminated soil is precisely 'non-contaminated urban soil' that was obtained from a garden (3°7′24.15′ N, 101°39′16.79′ E) at the University of Malaya in Malaysia. This non-contaminated urban soil was used to prepare the microcosms for bioremediation trials. It also served as the 'control'. Conversely, the contaminated soil was collected from a closed and non-sanitary landfill, specifically TBL. A soil auger was used during soil sampling. Briefly, the soil auger was inserted firmly into the soil while being rotated, until a 20 cm mark was reached. This was followed by further twisting before being pulled out. During soil sample collection, the contaminated soil was taken from the surface (0–20 cm) (3°13.78′ N, 101°39.72′ E) and then transferred into a soil bag. The collection of soil samples (replicates) was conducted as per the 2004 ASTM E-1197 guidelines, which have been recommended for performing microcosm tests for soil cores [14,15]. There was a replication (3 times) of sample collections to afford variability and homogeneity. After soil sample collection, the soil samples were air-dried while some portions were analyzed for the presence and levels of metals and metalloids using X-ray fluorescence spectrometry. Thereafter, a complete set of culturable microbes was isolated from the contaminated soil taken from TBL.

For leachate sampling, raw leachate was taken from the 4 different landfills five times (on various days). The samples were duplicated to ensure coherence in the study. While the leachate samples were collected from specified sites, such as seepages and overflows from BBL and TBL, other leachate samples were taken from pipelines directly related to the landfill cells at AHL and JSL. Physical, biological, and chemical characteristics were taken into consideration when performing laboratory analyses for the leachate samples from all 4 landfills. Parameters such as pH, color, odour, dissolved oxygen, total organic carbon (TOC), salinity, conductivity, suspended solids, total dissolved solids (TDSs), and a variety of other physicochemical parameters were determined for the collected leachate samples. The multipurpose Hach Sension 7 (HACH, Loveland, CO, USA) was used to measure salinity, conductivity, and TDSs, and the dissolved oxygen was measured using the DO 6+ Dissolved Oxygen meter (Eutech Instruments Pte Ltd., Singapur, Singapore) while the pH level and soil redox potential were measured using the HANNA HI8424 portable pH/mV meter (HANNA Instruments, Selangor, Malaysia). Thereafter, the leachate from JSL was used to contaminate the previously non-contaminated urban soil (taken from the garden at University of Malaya) for bioremediation experiments using microcosms. The leachate from JSL was selected out of the 4 investigated landfills because the highest concentrations of metals and metalloids were recorded for the leachate sample. The analyses of semi-volatile organic components such as phenol, alpha thujone, camphor, 1, 8-cineole and cresols, and monocyclic aromatic hydrocarbons (benzene, toluene, ethyl benzene, and xylenes) present in the leachate samples were conducted using gas chromatography–mass spectrometry.

Conversely, metals such as lead, cadmium, zinc, copper, manganese, iron, nickel, chromium, and others were measured via inductively coupled plasma mass spectrometry.

2.2. Isolation and Identification of Microbes

A complete set of culturable microbes was isolated from contaminated soil (soil collected from TBL) just before the start of bioremediation trials. Briefly, about 0.9% NaCl was mixed with 1 g of soil and the suspension was swirled for 2 h at 150 rpm using an orbit shaker (Lab-line 3521). Subsequently, serial dilutions were plated on nutrient agar (NA) and thereafter incubated for 48 h at 33 °C [14]. Single colonies were grown individually on freshly prepared NA to derive distinct pure cultures. The Biolog GEN III MicroPlate protocol was then used to identify the pure cultures [16,17].

For the identification, the cells were regrown every 16–24 h to avoid a loss of vigour and viability, which is synonymous with most organisms of the stationary phase. Using Protocols A (IF-A catalog number 72401) and B (IF-B catalog number 72403) at a turbidity range of 95–98% transmittance, each target cell was inoculated with inoculation fluid (IF). By using a cotton-tipped inoculator swab (catalog no. 3321), a 3 mm diameter area of cell growth was picked up from the surface of the agar plate and finally dipped into the desired IF. It was necessary to crush any cell clumps against the tube wall to ensure a uniform suspension.

Methodology for Biolog Identification

After culturing on an agar medium, the separated bacteria were suspended at the specified cell density in a unique "gelling" inoculating fluid. The MicroPlate was incubated to enable the formation of the phenotypic imprint, following the inoculation of 100 µL of the cell suspension into each well of the GEN III MicroPlate. Upon inoculation, all wells initially appeared colorless. Increased respiration occurs in the wells during incubation so that the cells can grow or use the carbon supply. The tetrazolium redox dye was reduced by increased respiration, resulting in the formation of a purple hue. Both the negative wells and the negative control well (A-1) without a carbon supply continued to appear colorless. Additionally, as indicated in Table S1, a positive control well (A-10) was used as a reference for the chemical sensitivity tests in columns 10–12. Following incubation, the purple wells' phenotypic fingerprint was compared to the vast species library of Biolog. The isolate is identified at the species level if a match is discovered.

2.3. Microbial Formulation for Bioremediation of Soil Contaminated with Toxic Metals

The isolated bacteria used in the present study are the complete set of culturable strains from the soil sample taken from TBL. Following the isolation and identification of microbes, a bioaugmentation process was developed to remediate soils contaminated with toxic metals. Nine bacterial strains were used for the bioaugmentation experiment. Before being inoculated in nutrient broth 'E' and brought to a stationary phase in a revolving shaker at 29 °C and 150 rpm, each strain was cultivated as a pure culture in NA plates at 33 °C for two days. To create inocula for bioaugmentation, each microbial strain was pooled in equal amounts (50 mL of each strain) and allowed to grow to 1.3 absorbance at 600 nm before being applied to the contaminated soil.

2.4. Experimental Setup for Bioremediation

The non-contaminated urban soil that was used to set up the microcosms was not sterilized prior to the experiment to represent the true condition of contaminated systems. Soil contamination was then performed according to the ASTM guidelines [15]. To achieve 10% v/w concentrations in 2 kg of soil, the characterized leachate was evenly distributed into each microcosm using potting bags. Specifically, leachate from JSL was used to artificially contaminate the soil. Before assembling the microcosms, the entire mass of the soil was contaminated with the leachate. It was thoroughly mixed and homogenized. This afforded homogenous initial concentrations in the soil and in each microcosm. Under different

conditions, 4 microcosms (groups) were constructed in triplicate as an indoor experiment at room temperature in an experimental laboratory that was well-aerated, with access to sunlight and normal airflow. With regard to how the 9 isolated bacterial species were classified, their grouping was based simply on how they responded to Gram staining. First, Group A represents contaminated soil amended with 6 bacteria strains, all Gram-negative except for *Microbacterium maritypicum*; second, Group B is indicative of soil amended with only 3 bacteria strains (all Gram-positive bacteria species); third, Group C denotes the treatment amended with all 9 isolated strains (both Gram-positive and Gram-negative species). Finally, the non-contaminated urban soil that was artificially contaminated with leachate, but without any introduction of isolated bacteria species, served as the control experiment. It was designated as Group D. The trial for bioremediation began with bioaugmentation using the microbial formula, three days after contamination [14].

Approximately 100 mL of the inocula (obtained from equal quantities of pooled distinct strains) with an average concentration of 3×10^9 CFU/g were added to microcosms A, B, and C. Soil moisture content was preserved by frequently spraying the soil with distilled water. Seepage was prevented by adding distilled water that was commensurate with keeping the soil moist. Every two days, approximately 100 mL of distilled water was added to the soil to maintain a moisture level between 60 and 65%. Over the course of the 100-day experiment, measurements of the redox potential, pH, and metal content of each treatment were performed every 20 days by periodic soil sampling and analysis.

2.5. Quantitative Assessment: Metal Analysis

Acid digestion was first performed to analyze the metal content of the soil samples. Briefly, HNO_3 and H_2O_2 were added to 0.5 g of soil sample before using the Anton Paar Multiwave 3000 microwave digester (Perkin Elmer, Waltham, MA, USA) for sample digestion [18,19]. Optima 5300 DV ICP-OES (Perkin Elmer, Waltham, MA, USA) was used to measure elemental concentrations [20,21]. An evaluation of a procedural blank was always conducted. All labware used in the experiment was soaked overnight in weak nitric acid before rinsing twice with deionized water. Duly replicated experiments were conducted for metal analysis.

2.6. Bacterial Count

The bacterial count was conducted every twenty days to determine how the microbial consortia changed in time throughout the entire 100-day bioremediation period. To count the bacteria, about 1 g of soil sample was combined with 10 mL of regular saline water (0.9% NaCl) as a stock. Using a Lab-line 3521 orbit shaker, the mixture was rapidly shaken for 3 h at 180 rpm. The resultant suspension was then serially diluted 20 times. Under aseptic circumstances, 0.1 mL of the dilutions was applied on freshly produced plate count agar. For a full day, the infected medium plates and related duplicates were incubated at 37 °C. A count of the developed colonies was taken after 24 h.

2.7. Determination of Metal Bioreduction

For a period of 100 days, concentrations of the toxic metals in the distinct microcosms were measured following analyses at 20-day intervals. Analysis of variance (ANOVA) was used to determine whether the results were significant at $p < 0.05$. The information was analysed to derive the percentage removal of toxic metals from each treatment. This is expressed in Equation (1).

$$\%\ \text{of contaminant (toxic metal) removal} = \left(\frac{C_{0(x)} - C_{F(x)}}{C_{0(x)}} \right) \times 100\% \quad (1)$$

where

$C_{0(x)}$ = initial concentration of contaminant in the soil;
$C_{F(x)}$ = final concentration of contaminant after treatment.

The rate constant of contaminant removal was obtained via the use of the first-order kinetic model;

$$K = -\frac{1}{t}\left(\ln\frac{C}{C_0}\right) \quad (2)$$

where

K = first-order rate constant for contaminant uptake per day;
t = time in days;
C = concentration of residual contaminant in the soil (mg/Kg);
C_0 = initial concentration of contaminant in the soil (mg/Kg).

2.8. Statistical Analysis

ANOVA with the least significant difference at a 0.05 p-value via SPSS software 21.0 was used to analyze the data.

3. Results and Discussion

The chemical parameters that were analyzed in the leachate samples are presented in Table S2 in the Supplementary Information.

3.1. Characterization of Leachate

The properties of the collected leachate samples differed among the four landfills, concerning quality based on varying concentrations across the measured parameters. Selected characteristics of the leachate samples obtained from the four different landfills are presented in Table 1.

Table 1. Physicochemical properties of landfill leachate samples.

Component	Unit	JSL	AHL	BBL	TBL	Standard Limits (EQA/EPA)
Color	-	Black	Bright Brown	Dark Brown	Bright Brown	-
Odor	-	Slightly ammoniac	Stench ammoniac	Ammoniac	Ammoniac	-
pH	-	7.35	8.2	7.1	6.8	6.0–9.0
Temperature	°C	27.5	29.5	28	27	40
Salinity	$^{0}/_{00}$	5.7	8.3	4.2	12	NA
Conductivity	mS/cm	10.04	20	14	34.6	NA
Turbidity	FAU	4150	108	274	130	NA
Dissolved Oxygen	mg/L	5.8	5.8	5.2	5.30	NA
BOD	mg/L	27,000	3500	259	127	20
COD	mg/L	51,200	10,234	985	482	400
BOD/COD	mg/L	0.53	0.34	0.26	0.26	NA
TDSs	mg/L	1730	830	860	2146	NA
Suspended Solid	mg/L	688	97	87	14	50
TOC	mg/L	380	110	70	42	NA
Grease and Oil	mg/L	48	7	3	4	5

Data represents mean values from n = 3; NA denotes not available.

Water quality and effluent discharge are influenced by color. Regarding the leachate characterization analysis presented in Table 1, the leachate sample from JSL had an apparent black color accompanied by a slight ammoniacal smell, but raw leachate samples from AHL, BBL, and TBL showed a brownish color (clearer) along with a stronger ammonia smell, especially AHL. Although color may not be a principal factor in quantifying the degree of toxicity of leachate, it can reflect the dissolved constituents of waste [22,23]. In this study, the variations observed in the selected landfill sites were closely associated with their operational status. While JSL and BBL are still active, AHL and TBL are closed. Currently, JSL and BBL still receive MSW and water-soluble compounds. Consequently, higher turbidity values (4150 and 274 FAU, respectively) and darker leachate colors were recorded for both landfills (Table 1). High levels of ammoniacal nitrogen (NH_3-N) were recorded in JSL (600 mg/L), TBL (630 mg/L), BBL (720 mg/L), and AHL (880 mg/L) (Table 2). Biotransformation, especially the hydrolysis and fermentation of organic nitrogen from MSW deposited in landfills, may be responsible for high NH_3-N levels. As biotransformation continues, the solubilization of soluble nitrogen increases, thereby increasing the concentration of NH_3-N in the leachate [24,25].

Table 2. Ionic components of the landfill leachate samples (mg/L).

Component	JSL	AHL	BBL	TBL	Standard Limits (EQA/EPA)
Chloride	4150	4150	4830	2780	250
Sulphate	54.89	37.1	92.3	65.3	250
Phosphate	113	70.2	100	92	5
Nitrate Nitrogen	38.6	29.1	40.1	35.2	10
Nitrite Nitrogen	4.8	2.7	23.3	20.1	1
Ammoniacal nitrogen	600	880	720	650	5

Concentration of all nitrogen forms is expressed as mg/L-N.

Since there is no mechanism for the breakdown of NH_3-N under methanogenic conditions, it is difficult to reduce the NH_3-N content in landfills unless this is performed through leaching [26,27]. Ammoniacal nitrogen has been identified as the most significant long-term component of leachate [10,28].

Notably, the levels of soluble metal in the active sanitary landfills (JSL and BBL) are higher than those in the older (closed) landfills (AHL and TBL) (Table 3). This agrees with findings reported in similar studies [3,29].

Table 3. Metal components of the landfill leachate samples (mg/L).

Component	JSL	AHL	BBL	TBL	Standard Limits (EQA/EPA)
Chromium	25.27	0.11	17.3	6.2	0.05
Copper	3.59	<0.001	2.62	0.5	0.2
Nickel	19.50	0.29	12	0.85	0.2
Zinc	827.7	0.1	236	24.3	2.0
Manganese	540.6	0.12	5.1	3.1	0.2
Iron	97.76	3.10	7.13	4.89	5.0
Potassium	530	440	530	390	NA
Magnesium	11.4	20.3	25.5	20.4	NA
Sodium	58.7	48.6	40.3	35.2	NA

NA—not available.

None of the noteworthy harmful organochlorine pesticides, such as DDT, Dieldrin, 4, 4-DDE, and Chlorpyrifos, have concentrations of more than 0.01 µg/L (Table 4). The controlled usage and disposal of products containing such chemical components may be the reason for the minimal concentration of such chemical components.

Table 4. Organochlorine pesticide components of the landfill leachate samples (µg/L).

Component	JSL	AHL	BBL	TBL	Standard Limits (EPA)
Aldrin	<0.01	<0.01	<0.01	<0.01	NA
α-BHC	<0.01	<0.01	<0.01	<0.01	NA
β-BHC	<0.01	<0.01	<0.01	<0.01	NA
χ-BHC	<0.01	<0.01	<0.01	<0.01	NA
δ-BHC	<0.01	<0.01	<0.01	<0.01	NA
4,4-DDE	<1	<1	<1	<1	NA
4,4-DDT	<1	<1	<1	<1	NA
DDT	<0.01	<0.01	<0.01	<0.01	0.044
Dieldrin	<0.01	<0.01	<0.01	<0.01	NA
Endosulfan I	<1	<1	<1	<1	0.08
Endosulfan II	<1	<1	<1	<1	0.08
Endosulfan Sulfate	<1	<1	<1	<1	0.08
Endrin	<0.1	<0.01	<0.01	<0.01	2
Endrin Aldehyde	<0.1	<0.01	<0.01	<0.01	NA
Hepatchlor Epoxide	<0.1	<0.01	<0.01	<0.01	0.2
Heptachlor	<0.01	<0.01	<0.01	<0.01	0.4
Lindane	<1	<1	<1	<1	0.2
Methoxychlor	<1	<1	<1	<1	40

NA—not available.

Other relevant data related to analyses of the leachate samples, such as those for the monocyclic aromatic hydrocarbons and semi volatile organic components, are presented in the Supplementary Information (Tables S3 and S4). With regard to the characterization of a soil sample that was collected from TBL, Table S5 shows that the levels of heavy metal(loid)s in the soil are higher than the limits of 0.6, 10, 1.6, 2, and 10 mg/kg for Zn, Cu, Cr, Pb, and Ni, respectively, as recommended by the World Health Organization (WHO) [30]. The metal concentrations in soils taken from unlined landfills, such as TBL, which are considered non-sanitary dumps usually indicate the types of waste that were disposed of. Hazardous waste, both industrial and household, is frequently disposed of in landfills in Malaysia. This could be a possible reason for the higher-than-average concentrations of metals and metalloids reported for TBL (Table S5).

3.2. Removal of Toxic Metals from Leachate-Contaminated Soils

In the present study, microbes were isolated from the soil collected from TBL (a landfill that was originally contaminated with leachate). The isolated microbes were then used for bioaugmentation as a method for remediating soil microcosm systems (previously non-contaminated urban soil) that were contaminated with leachate for bioremediation trials. The leachate taken from JSL was used to induce metal contamination in the microcosms. The results of toxic metal removal from leachate-contaminated soils are presented in this section.

3.2.1. Microbial Isolation from Originally Contaminated Soil

The original leachate-contaminated soil was characterized for bacteria based on separation and identification. Nine bacteria that were isolated from the leachate-contaminated soil are listed in Table 5. Both Gram-positive and Gram-negative bacteria were isolated. Even though some of the identified microbes may not be depicted in the literature as bioremediation enhancers, their presence in leachate-contaminated soil sparks curiosity about their existence and role in the contaminated environment. Notably, it is observed that some microorganisms possess the capacity to survive in environments contaminated with toxic metals and even promote changes that minimize their toxicity. This suggests that they could be effective in bioremediation.

Table 5. Isolated microbes and their distribution in different microcosms for bioaugmentation.

Treatment A	Treatment B	Treatment C	Treatment D (Not Amended)
-	*Bacillus thuringiensis*	*Bacillus thuringiensis*	-
Psuedomonas putida biotype B	-	*Psuedomonas putida biotype B*	-
Stenotrophomonas maltophilia	-	*Stenotrophomonas maltophilia*	-
Flavimonas oryzihabitans	-	*Flavimonas oryzihabitans*	-
-	*Lysinibacillus sphaericus*	*Lysinibacillus sphaericus*	-
Acinetobacter schindleri	-	*Acinetobacter schindleri*	-
Brevundimonas vesicularis	-	*Brevundimonas vesicularis*	-
Microbacterium maritypicum	-	*Microbacterium maritypicum*	-
-	*Rhodococcus wratislaviensis*	*Rhodococcus wratislaviensis*	-

Treatment A represents 6 isolated bacterial species, mainly Gram-negative; Treatment B signifies 3 bacterial species that are Gram-positive; Treatment C is indicative of all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Treatment D serves as the control experiment with no introduction of isolated bacterial species.

The Gram-positive bacteria in the group, according to Table 5, were *Lysinibacillus sphaericus*, *Rhodococcus wratislaviensis*, *Bacillus thuringiensis*, and *Microbacterium maritypicum*. Gram-negative bacteria were identified as *Brevundimonas vesicularis*, *Stenotrophonomas maltophila*, *Flavimonas oryzihabitans*, *Acinetobacter schindleri*, and *Pseudomonas putida* biotype B.

Bacillus thuringiensis is a prevalent soil bacterium; it is related to the presence of *Bacillus cereus*. *Bacillus cereus* is often seen as a biochemical indicator for concealed mineralisation [31]. Despite its high similarity with *B. cereus*, *B. thuringiensis* is widely known for its insecticidal and nematicidal properties [32,33]. Bio-control agents derived from *B. thuringiensis* are widely used in agriculture and medicine for eradicating pests [34–36]. *Bacillus* species have been reported to be useful for the removal of toxic metals from contaminated soils [37,38], and their presence in soil has been linked with metal abundance [39,40].

Likewise, *Pseudomonas putida biotype B* is also an organism that is common in polluted soil [41,42]. Though the organism may have some clinical implications, especially in pathogenicity, its resistive nature to pollution, especially to some toxic metals from aqueous solutions, may have influenced its presence in the leachate-contaminated soil [43,44].

Stenotrophomonas maltophila, a Gram-negative aerobic bacterium, was also isolated from the leachate-contaminated soil. It is pervasive in the environment [45]. It inhabits the rhizosphere of plants such as potatoes, maize, wheat, oilseed, cucumber, and oat [46,47]. It can degrade xenobiotic compounds [48,49]. It can detoxify high molecular weight polycyclic aromatic hydrocarbons [45,50].

Lysinibacillus sphaericus is another Gram-positive bacterium that was isolated in this study. There have been reports of its existence in contaminated areas and wastewater [51,52]. Its significance in this work can be supported by [53], which found that isolated *L. sphaericus*

have a bioremediation effect by acting as a metal binding site, and [54] which showed the organism's ability to reduce and oxidize manganese in water.

The aerobic, irregularly rod-shaped and Gram-positive bacteria *Rhodococcus wratislaviensis* was also isolated from the leachate-contaminated site. It is predominant in soils [55,56]. Ref. [57] reported that the *R. wratislaviensis* strain that was isolated from forest soil was able to degrade nitroaromatic compounds. The isolation of the microbe in this work may be significant in terms of its ability of direct degradation or symbiotic potential to enhance bioremediation, as depicted in the studies [58,59].

It is important to note that despite the isolation of only nine bacteria species, the high resistive nature of microbes to leachate-contaminated sites in general, and toxic metals in particular, abounds. Therefore, considering this potential relevance of microorganisms, the present study further grouped the microbes into different classes, as shown in Table 5. The groups of isolated bacteria were used for the bioaugmentation of leachate artificially contaminated soil for bioremediation tests.

3.2.2. Bioreduction of Metals

The concentrations of toxic metals were ascertained before and after treatment (Table 6). Following bioremediation, the metal concentrations were determined based on the metal available in the soil after treatment (residual concentration).

Table 6. Initial and final concentrations of selected toxic metals from the bioremediation of leachate-contaminated soil.

Metals	Initial Concentration (mg/kg)	Final Concentrations in mg/kg (Average Residual and Standard Deviation Values) across Different Groups			
		A	B	C	D
Cr	0.265	0.05 ± 0.017	0.05 ± 0.017	0.09 ± 0.02	0.10 ± 0.012
Fe	154	63.74 ± 17.8	51.13 ± 4.00	67.33 ± 21.39	80.33 ± 8.96
Zn	2.71	1.09 ± 0.016	0.72 ± 0.09	1.16 ± 0.15	1.41 ± 0.08
Cu	0.241	0.07 ± 0.015	0.03 ± 0.015	0.09 ± 0.015	0.11 ± 0.015
Mn	1.29	0.48 ± 0.13	0.45 ± 0.17	0.46 ± 0.13	0.98 ± 0.049
Pb	2.068	0.79 ± 0.16	0.60 ± 0.17	0.84 ± 0.18	1.21 ± 0.2

n = 3. Group A represents soil amended with 6 isolated bacterial species, mainly Gram-negative; Group B represents soil amended with 3 bacterial species that are Gram-positive; Group C is indicative of soil amended with all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Group D serves as the control experiment with no introduction of isolated bacterial species.

Upon bioaugmentation of the leachate-contaminated soil, reductions in the concentrations of the toxic metals were observed across all days of biomonitoring and treatments (Groups A–D). The initial mean concentration of each metal in the leachate-contaminated soil was the same for all the treatments (Groups A–D). The residual mean concentrations following 100 days of biomonitoring are shown in Table 6. The metals investigated in the present study are discussed individually across time and treatment groups.

The concentration of the toxic metal in the leachate-spiked soil at day 1 is denoted as "0", followed by intermittent monitoring for the next 100 days at 20-day intervals. Therefore, the last monitoring was represented as "5". According to the one-way analysis of variance (ANOVA) for Pb, there were significant differences between the initial day of contamination (day 1) and the final periods of monitoring. The level of significance was higher with the amended (inoculated) treatments than with the control experiment (p = 0.002). This is probably due to the role played by the introduced microorganisms. This agrees with published findings that have highlighted the effectiveness of microbes in the bioremediation of metal-contaminated soils [60,61].

The highest removal of Pb was recorded in Group B (Table 6). This was expected for Group C, where all 9 isolated bacteria species were utilized. The trend observed for Group B (with only three Gram-positive bacteria) may have occurred due to the interactions that

exist among microbes when concentrations are manipulated. For instance, *B. thuringiensis*, which is present in two of the inocula-amended microcosms (Groups B and C), has been found to possess a high capacity for toxic metal removal. Ref. [37] reported that about 77% of Pb was removed from an extract medium of a mine tailing that contained 100 mg/L of Pb. In spite of the presence of *B. thuringiensis* in Group C, the removal of Pb was more evident in treatments A and B; therefore, the order of Pb removal across the groups was D < C < A < B (Table 6).

Figure 1 shows the degree to which Pb was removed from the different treatments. It is observed that while about 71% Pb was removed in Group B in 100 days, only about 42% was removed in the control experiment (Group D). Possibly, natural attenuation may have played a significant role with respect to the removal of Pb in Group D. After all, the soil was not sterilized or autoclaved; therefore, naturally inherent microbes may have some effect, though to a reduced extent in comparison to other treatments. Using a one-way ANOVA, F = 15.566, the difference in Pb removal between Groups B (treatment that showed highest removal) and D (control experiment showing least removal) was significant at p (0.017) < 0.05. Therefore, a significant reduction in the concentration of Pb was observed with the introduction of inocula into the leachate-contaminated soil.

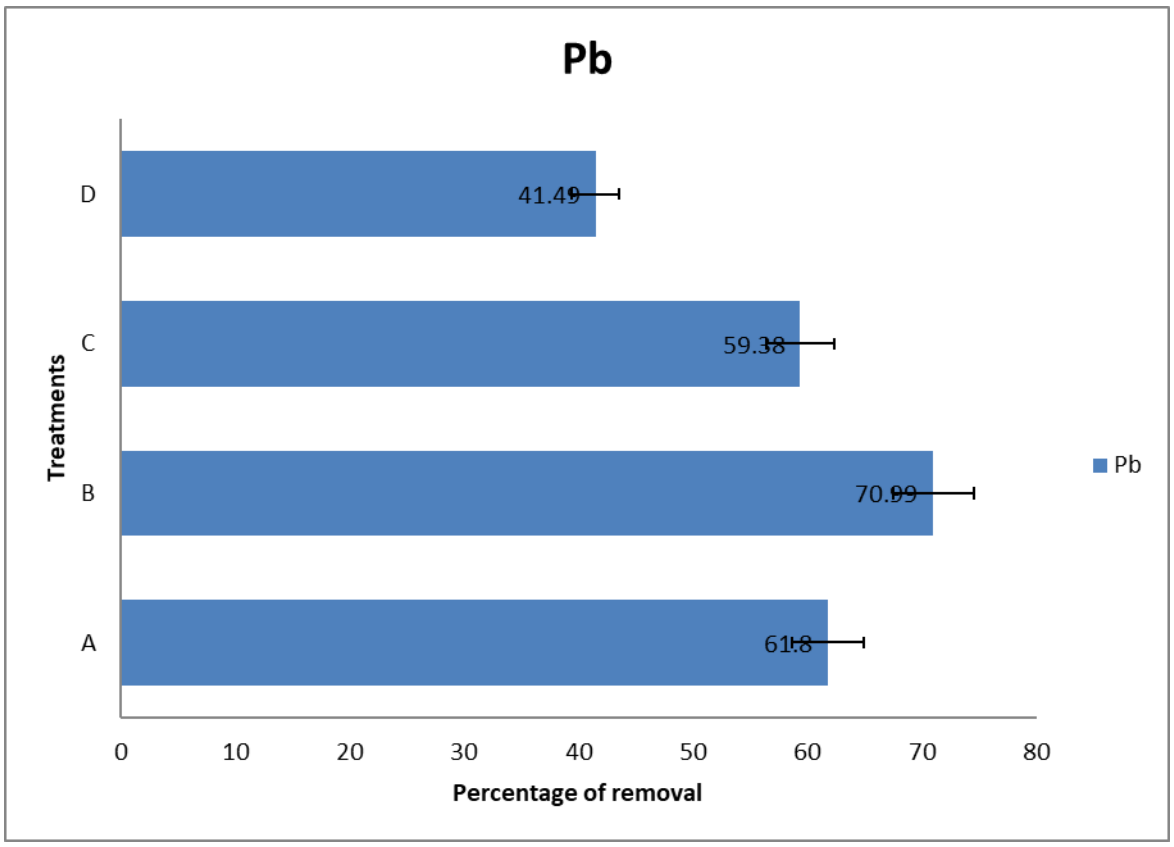

Figure 1. Percentage of lead (Pb) removed during bioremediation. (Error bars represent the standard deviations of triplicates of each sample. Treatment A represents 6 isolated bacterial species mainly Gram-negative; Treatment B signifies 3 bacterial species that are Gram-positive; Treatment C is indicative of all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Treatment D serves as the control experiment with no introduction of isolated bacterial species).

It can be observed that the amended soils (Groups A, B, and C) reduced the Mn content three times more than the non-amended soil (Group D) (Figure 2; Table 7). It can be inferred that microbial activities could initiate metabolic reactions favorable for the biodegradation of Mn. This suggestion is in consonance with the findings reported in [62,63].

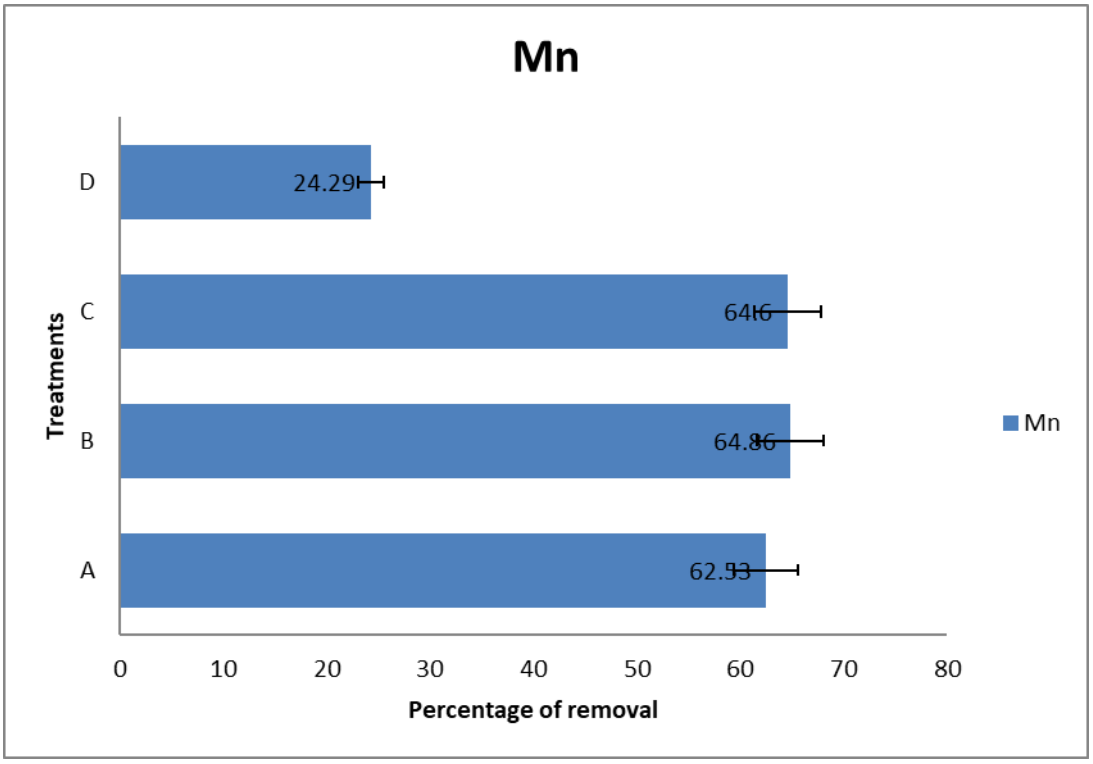

Figure 2. Percentage of Mn removed during bioremediation.

Table 7. Removal rate constant (k) of toxic metals.

	Daily Amount of Metal Degraded (Day^{-1}) from Microcosms			
	Group A	Group B	Group C	Group D
Fe	0.0089	0.0111	0.0082	0.0065
Pb	0.0097	0.0124	0.0089	0.0053
Zn	0.0092	0.0131	0.0084	0.0065
Cr	0.0167	0.0167	0.0108	0.0097
Cu	0.0124	0.0212	0.0099	0.0078
Mn	0.0099	0.0105	0.0099	0.0027

Group A represents soil amended with 6 isolated bacterial species, mainly Gram-negative; Group B represents soil amended with 3 bacterial species that are Gram-positive; Group C is indicative of soil amended with all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Group D serves as the control experiment with no introduction of isolated bacterial species.

In Groups A, B, C, and D, the mean residual concentrations of Cu were 0.07, 0.03, 0.09, and 0.11 (mg/kg) after 100 days of biomonitoring (Table 7). Figure 3 shows that the quantity of Cu that was removed in Group B is slightly greater than 86%, in comparison to 69%, 64%, and 52% recorded for treatments A, C, and D, respectively. According to the one-way ANOVA that was performed, there was a significant difference between B and D (p = 0.003) and even with the other groups, for instance, AB (p = 0.033) and BC (p = 0.013). It is probable that *B. thuringiensis* may have influenced the rate of reduction, especially in treatments B and C where it was present (Table 6). This conclusion is supported by the findings in the study [64], where the potential of *B. thuringiensis* in the removal of Cu from soil contaminated with industrial effluent was reported. The optimal removal of Cu from contaminated soil recorded in Treatment B (Figure 3, Table 6) suggests that there may be a synergistic effect occurring among the selection that includes *B. thuringiensis*, *L. sphaericus*, and *R. wratislaviensis*.

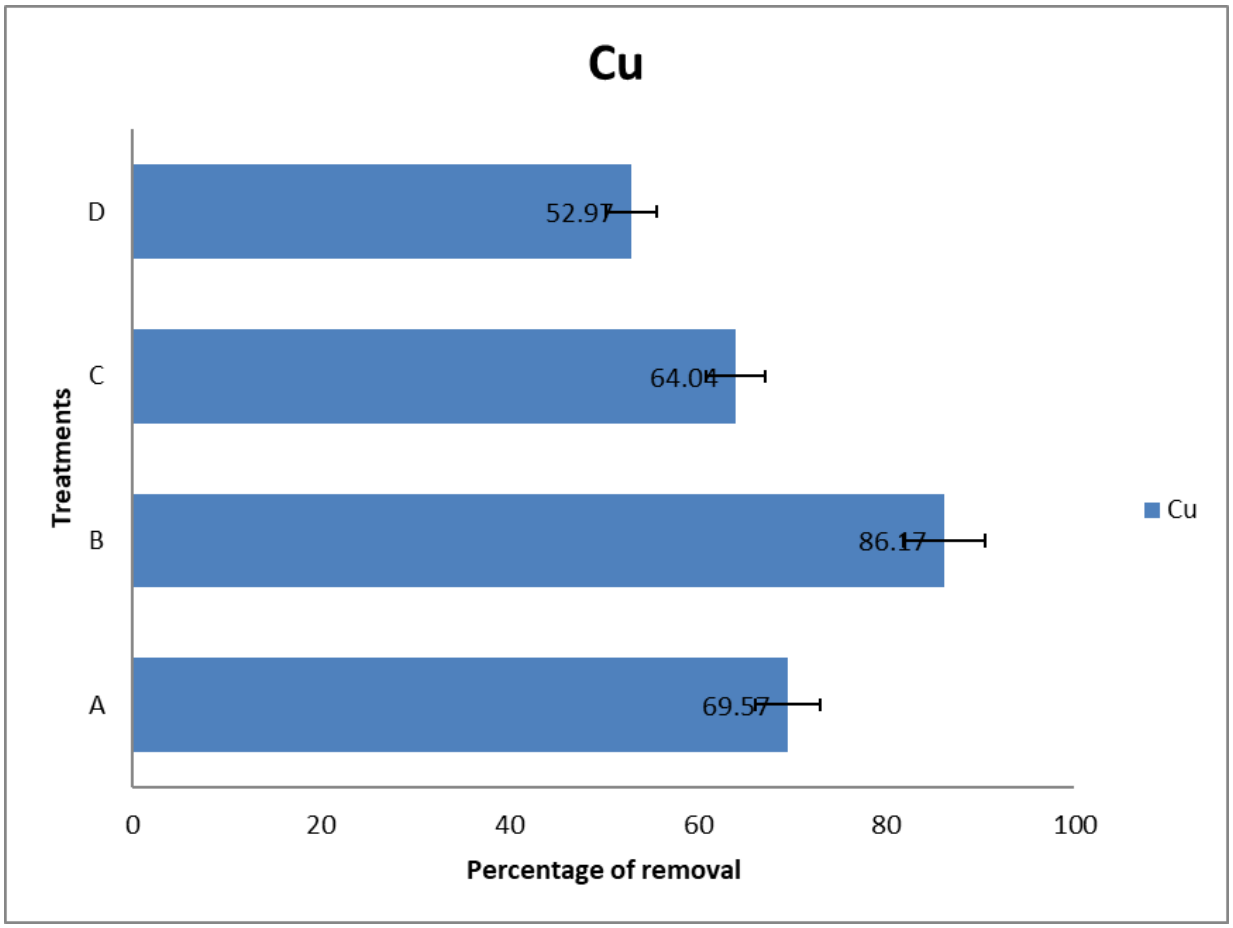

Figure 3. Percentage of Cu removed during bioremediation.

The highest removal of Zn (73.4%) was also recorded in Treatment B (Figure 4). These bacteria species found across treatments A, B, and C, namely *B. thuringiensis*, *P. putida biotype B*, and *M. maritypicum* may have been significantly involved in the bioremediation of Zn. Several studies have found them to be effective in the removal of Zn. According to the study in [65], about 14–68% of Zn was removed from polluted residue via *Microbacterium* sp. Also, ref. [66] reported the effective removal of Zn in the presence of *P. putida* strains, MH3, MH6, and MH7. Similarly, *B. thuringiensis* GDB-1 was able to remove about 64% of Zn in a metal-contaminated site [37]. Therefore, a difference with respect to removal efficiency for Zn is observed between the inoculated microcosms and the control experiment (Figure 4).

There was also better performance in terms of the removal of Fe in the amended treatments than in the control experiment, with the highest removal observed in Group B (Figure 5; Table 7).

In Figure 6, Groups A and B exhibited the same degree of Cr reduction (81%) compared to 67% and 64% recorded for treatments C and D, respectively. This indicates that while the natural attenuation of Cr from leachate-contaminated soil is possible (Treatment D), improved removal can be achieved via bioaugmentation using the isolated microbes. The similarity observed between treatments A and B (Figure 6) may be related to the presence of specific bacteria species that are known to be able to degrade Cr in contaminated media. Such microbes include *Stentrophomonas maltophilia* [67]; *Pseudomonas* sp. [68,69]; *Bacillus* sp. [68,70]; *Acinetobacter* sp. [71]; *Rhodococcus* sp. [69]; and *L. sphaericus* [72].

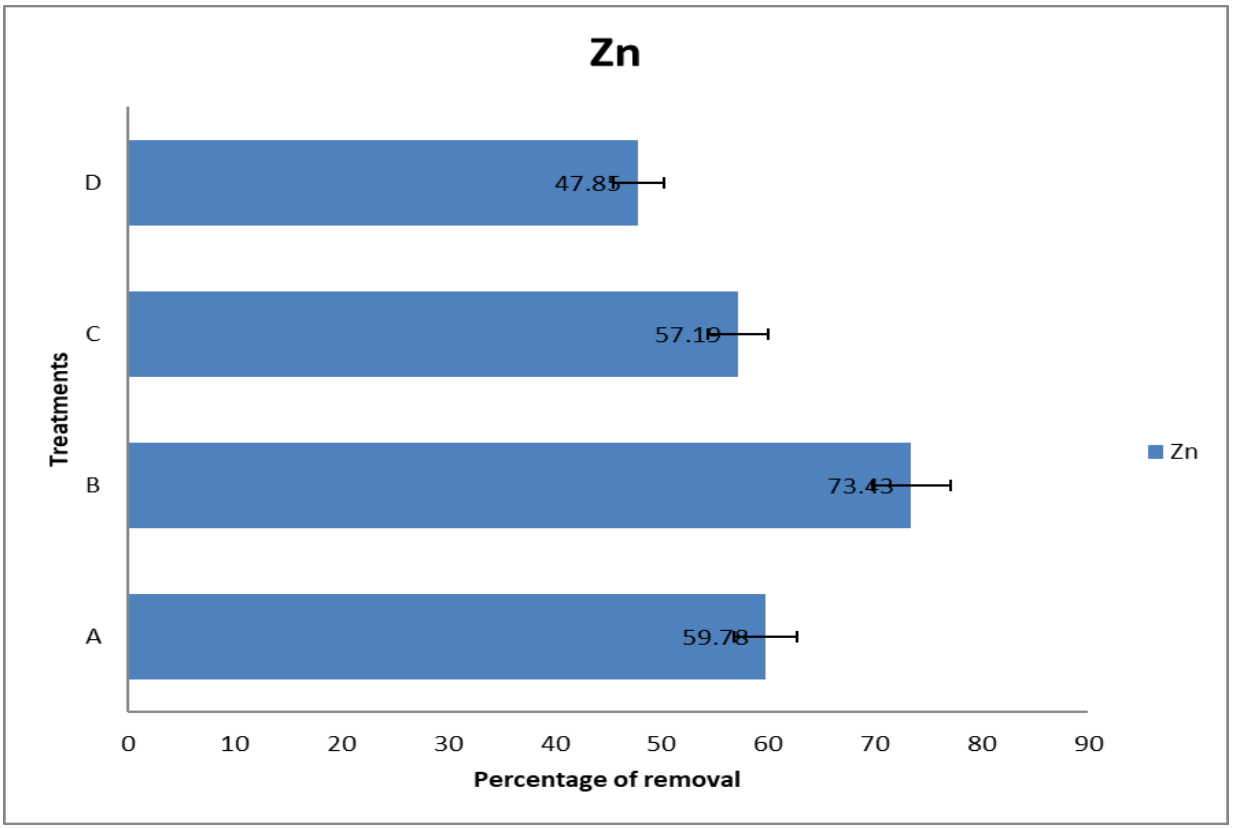

Figure 4. Percentage of Zn removed during bioremediation.

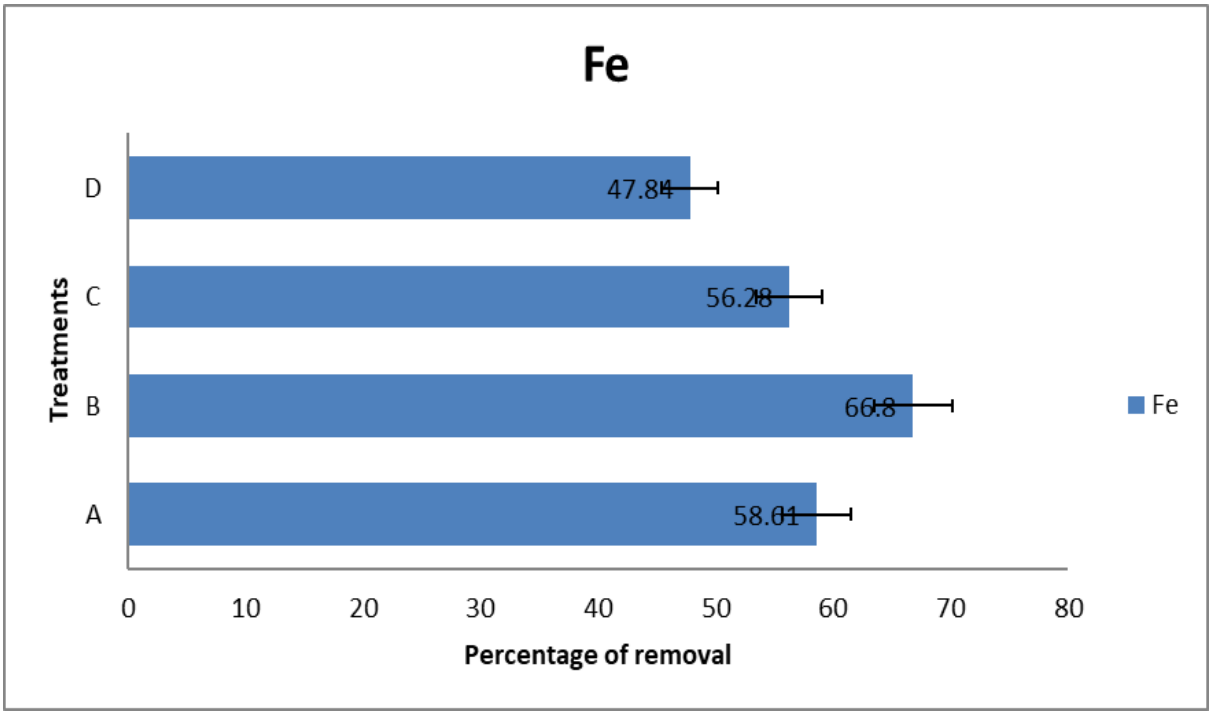

Figure 5. Percentage of Fe removed during bioremediation.

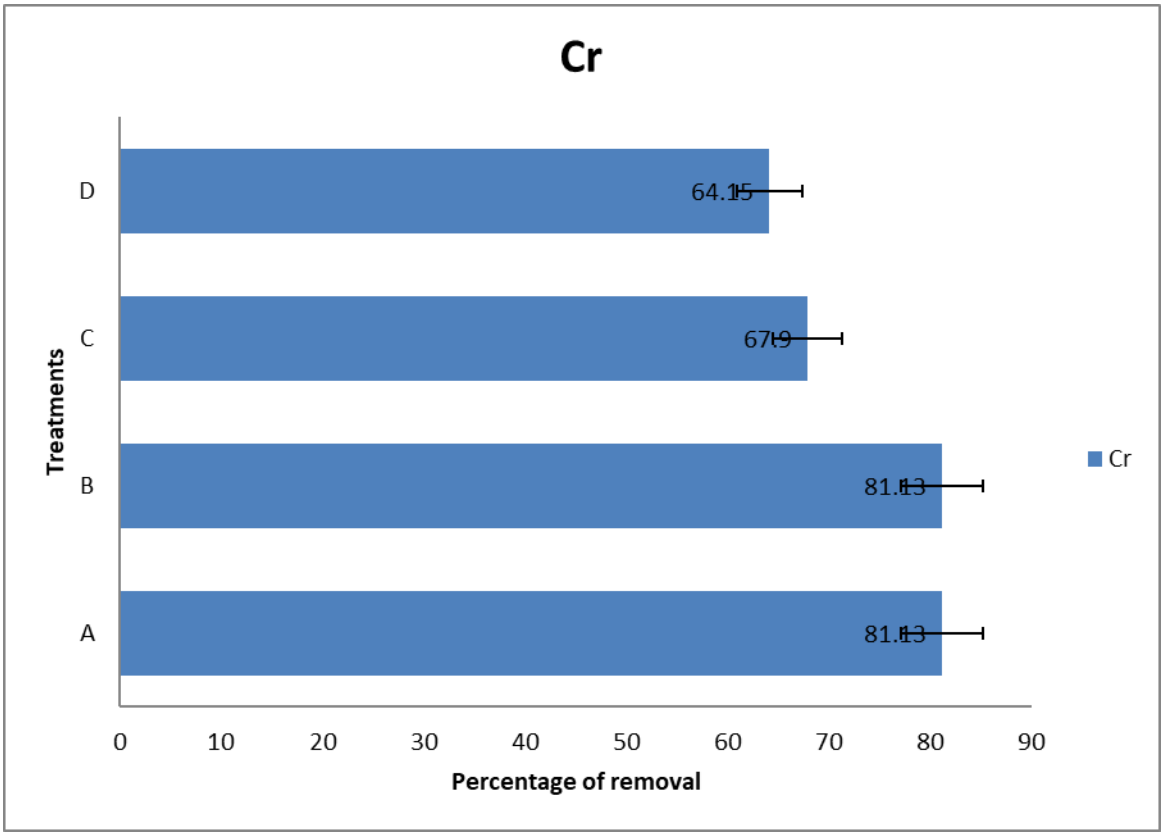

Figure 6. Percentage of Cr removed during bioremediation.

3.2.3. Rate Constant of Toxic Metal Removal

In the present study, we also studied the removal rate constants of the toxic metals (Pb, Mn, Cu, Zn, Fe, and Cr) per day in the different microcosms via the first-order kinetic model. This elucidates the removal ability of the microbial combinations daily, as it relates to each specific metal. This was then compared to the control experiment. It is evident from Table 7 that the rate constant for the removal of Cu in the contaminated soil that was amended with only three bacteria species (*B. thuringiensis*, *L. sphaericus*, and *R. wratislaviensis*) (Group B) within the 100-day study is the highest among the selected metals. It is possible that since the C-terminus of the S-layer protein SbpA of *L. sphaericus* possess a hex-histidine tag (His_6—tag), this hex-histidine tag (metal binding property) is better expressed when it is combined with only *B. thuringiensis* and *R. wratislaviensis* (Group B) unlike with eight other species in Group C. Hence, the bioremediation edge was observed for Group B throughout the study (Table 7).

The results from Group D show that some metals within the group may undergo natural remediation especially when contaminated soil is left undisturbed (Table 7). Conversely, other metals in Group D that exhibited low removal rates in comparison to Groups A, B, and C could be a result of the inability of the pre-existing microbes to be self-sufficient regarding the bioremediation of contaminated soil. It is apparent that the amended soils showed greater bioremediation ability than the control experiment. However, the amendments prioritized the removal of toxic metals differently. For instance, Groups A and C prioritized Cr removal at 0.0167 day^{-1} and 0.0108 day^{-1}, respectively, while Group B prioritized Cu removal (0.0212 day^{-1}) (Table 7). Therefore, it can be inferred that complex microbial interactions exist within the different microcosms. Consequently, blending the microbes affords the maximum removal of toxic metals as is evident in Group B.

Specifically, with respect to the impact of leachate on the soil after the artificial contamination of the non-contaminated soil that was obtained from a garden, soil chemical characteristics such as soil pH and soil redox potential were monitored throughout the

100-day bioremediation period. Firstly, the pH of the soil from the garden at the University of Malaya, Malaysia, was found to be 7.52 before leachate contamination. After artificial contamination using leachate, at the onset of the bioremediation trials (day 0), the pH of the soils (Groups A-D) was found to be 8.2 (Figure 7). It is worth mentioning that the leachate used for artificial contamination was obtained from JSL, which showed neutral–slightly alkaline pH, typical of an old landfill in its methanogenic phase. The pH of the artificially contaminated soil (8.2) was conducive to the growth of microbes. Regarding the pH changes in the soil during the bioremediation study, on day 20, the pH range for treatments A, B, C, and D was 7.65, 7.3, 7.47, and 7.68, whereas on day 100, the pH range was 8.07, 8.01, 8.11, and 8.21 for treatments A, B, C, and D, respectively (Figure 7). The variation in soil pH could be due to binding of metals upon the introduction of bacterial inoculum. The optimal pH condition for microbial growth and maximum removal of chromium ion (Cr (VI)) was found to be in the pH range of 7–8 in the study in [67]. This study corroborates the findings from our study where the bioreduction of hazardous metals and metalloids has also been observed at a similar pH range (neutral–slightly alkaline).

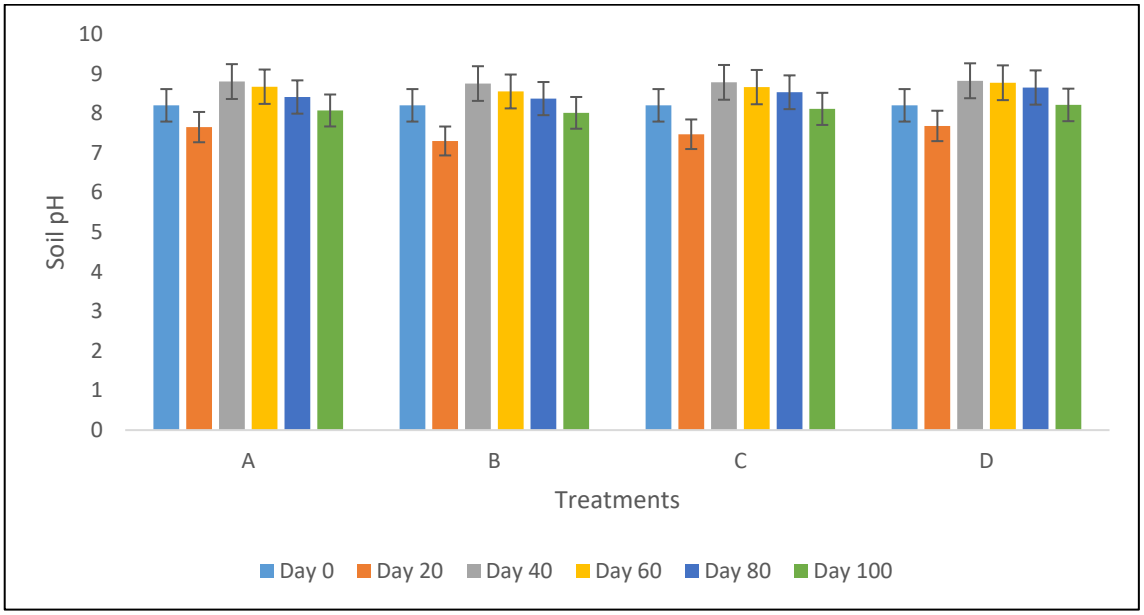

Figure 7. Soil pH across time for different treatments (A–D) during bioremediation trials. (Treatment A represents soil amended with 6 isolated bacterial species mainly Gram-negative; Treatment B represents soil amended with 3 bacterial species that are Gram-positive; Treatment C is indicative of soil amended with all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Treatment D serves as the control experiment with no introduction of isolated bacterial species).

Also, in the course of the bioremediation process, the soil redox potential was evaluated for the soil(s) subjected to remediation. Figure 8 shows that there is a mild but progressive increase in the redox potential (mV) from day 20 to day 100, except with a slight dip at day 60, for all the treatments. For instance, the redox potential ranged from 207.13 mV at day 20 to 261.73 at day 100, and from 186.73 mV at day 20 to 232.43 mV at day 100 for treatments A and B, respectively (Figure 8). The increase in redox potential during bioremediation trials is indicative of oxidizing conditions [73]. For example, a metal ion such as Fe^{2+} under neutral pH and oxidizing condition could be converted to Fe^{3+} in the form of iron hydroxide, $Fe(OH)_3$, which then precipitates out of the solution. The findings from this study for both soil pH and soil redox potential are indicative of neutral–slightly alkaline pH (pH 7–8) and oxidizing conditions. Both conditions enable the alteration of oxidation states of metal ions and the occurrence of metal transformations [73,74].

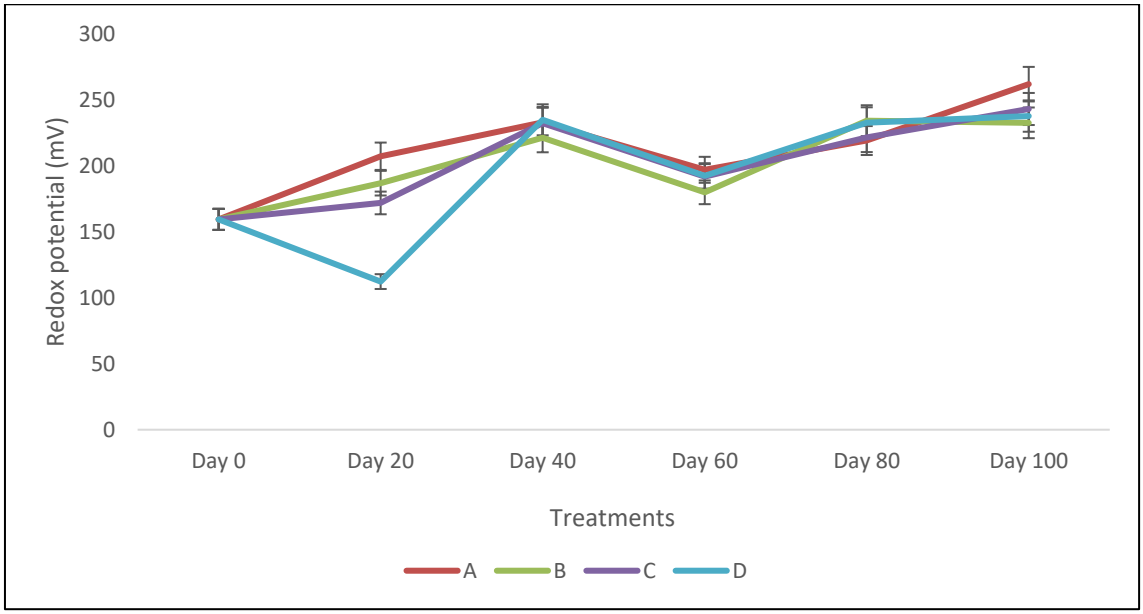

Figure 8. Soil redox potential across time for different treatments (A–D) during bioremediation trials. (Treatment A represents soil amended with 6 isolated bacterial species mainly Gram-negative; Treatment B represents soil amended with 3 bacterial species that are Gram-positive; Treatment C is indicative of soil amended with all the 9 isolated bacterial strains including both Gram-negative and Gram-positive species; and Treatment D serves as the control experiment with no introduction of isolated bacterial species).

In addition, besides the analyses of the toxic metals' concentrations, to further understand the interaction of the inoculated bacteria with the contaminated soil, the bacterial count was evaluated. During the 100-day remediation period, the bacterial count was performed every twenty days. The bacterial count over time for treatments A–D is presented in Figure S1. It is crucial to remember that low inocula can hinder the survival of bioaugmented bacteria when considering the potential effects of other microbes. This study employed high inocula because bioaugmented bacteria colonization is a vital component of bioaugmentation success. To be precise, the inoculum in each treatment constituted roughly 3×10^9 CFU/g. Understanding the distribution of bacteria in a bioremediation setup is essential because the number of bacteria in the soil is indicative of the survival of microbes in metal-contaminated soils. The bacterial count showed a fluctuating trend throughout the course of the 100 days. For the first 20 days, the bacterial count ranged from 3.0×10^{11} to 6.5×10^{10} CFU/g for the amended treatments (Figure S1). Ref. [75] observed similar patterns in the number of bacteria throughout their bioremediation studies. The rise in the number of bacteria at days 40 and 60 is most likely due to the availability of nutrients in the soil. The advent of conditions that are conducive to cell duplication may have also contributed to an increase in the number of bacteria. As the experiment came to an end (Day 100), there was a noticeable decrease in the number of bacteria for Groups A–D (Figure S1); depicting the extent to which natural and inocula bacteria bio-remove hazardous metals.

The findings in this study can be corroborated by the study in [76]. In that study, indigenous bacteria that were isolated from a tannery sludge in India were able to remove Zn (69.9%) and Mn (78.4%) from metal-contaminated soil. In the present study, the His_6—tag at the C-terminus of the S-layer protein of *L. sphaericus* may have enhanced its metal binding effect in the presence of *R. wratislaviensis* and *B. thuringiensis*. The results in the study in [77] also lend credence to the findings of the present study.

4. Conclusions

A significant finding in this study is that a bacterial consortium composed of only three strains (all Gram-positive bacteria) was found to be more effective in the remediation of leachate-metal-contaminated soil, based on the bulk of the results. It can be inferred that the isolation, identification, strain selection, inoculation, colonization, and effect processes are more pivotal compared to a treatment with a greater number of bacterial species and hence more variety. The availability of oxygen, potential environmental interference from sunlight, rain, temperature, microbial competition, soil texture and wetness, concentration, toxicity, the bioavailability of target pollutants, and the presence of co-contaminants are some of the aspects that will be optimized during future studies. Overall, microbial remediation is a promising and environmentally friendly approach to addressing the issue of the presence of toxic metals in leachate-metal-contaminated soils.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/soilsystems8010033/s1, Table S1: Layout of Assays for MicroPlate (GEN III); Table S2: Chemical components analyzed in the leachate samples; Table S3: Monocyclic Aromatic Hydrocarbon Components of Leachate Samples (mg/L); Table S4: Semi-volatile Organic Components of Leachate Samples (mg/L); Table S5: Characterization of soil sample collected from Taman Beringin Landfill; and Figure S1: Bacterial counts during the 100 days of biomonitoring.

Author Contributions: Conceptualization, C.E. and F.S.H.; Methodology, C.E. and J.B.; Software, J.B.; Validation, C.E. and F.S.H.; Investigation, C.E., A.P. and F.S.H.; Resources, F.S.H.; Writing—Original Draft, C.E. and P.O.-O.; Writing—Review and Editing, C.E. and P.O.-O.; Supervision, C.E., A.P. and F.S.H.; Funding Acquisition, C.E. All authors have read and agreed to the published version of the manuscript.

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