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Amplicon Based Insights into Bacterial Community Assembly of Dominant Taxa in Biodegradation of Crude Oil Sludge Composting Microcosms with Animal Manures

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Abstract

Crude oil sludge, a hazardous byproduct of petroleum refining, poses persistent ecological risks due to its complex matrix of polycyclic aromatic hydrocarbons (PAHs). Although composting with organic amendments can stimulate microbial degradation of these compounds, the ecological processes governing bacterial community assembly characterisation in such systems remain paramount in current bioremediation technology. Four animal manure amendments investigated: cow, swine/pig, horse, and poultry, shaped bacterial community structure, diversity, and functional potential within crude oil sludge co-compost microcosms over a 300-day composting period. Amplicon-based profiling of the 16S rRNA gene (Illumina MiSeq, V1–V3 hypervariable regions) identified 359 bacterial genera spanning 17 phyla across five treatments. Manure type emerged as the dominant ecological driver of community composition. Cow manure supported the highest microbial richness (Chao1 = 422.60) and diversity (Shannon H = 3.726), whereas pig and poultry amendments selectively enriched known PAH-degrading specialists, including *Pseudomonas*, *Sphingomonas*, and *Burkholderia-Paraburkholderia*. Functional prediction using PICRUST2 (NSTI = 0.02–0.16) revealed pathways for degradation and aromatic compound metabolism. Culture-based validation confirmed that dominant isolates harboured the catechol 2,3-dioxygenase (*cbzE*) gene and demonstrated active aromatic ring cleavage. Long-term PAH monitoring documented up to 99.9% removal of PAHs in pig and poultry manure treatments. These findings reveal a trade-off between microbial richness and functional specialisation, demonstrating that manure-driven ecological selection assembles hydrocarbon-degrading consortia, and provide a scalable framework for compost-based bioremediation of petroleum-contaminated environments.

Keywords: *Amplicon metagenomics, Bacterial community assembly, Co-composting, Crude oil sludge, Microbial ecology, PAH biodegradation, Hydrocarbon-degrading bacteria*

1. Introduction

Crude oil sludge accumulates during petroleum extraction, transport, and refining and contains a complex matrix of polycyclic aromatic hydrocarbons (PAHs), heavy metals, and hydrophobic organic compounds [1]. The structural recalcitrance and hydrophobicity of high-molecular-weight (HMW) PAHs, arising from their fused aromatic rings, render them resistant to abiotic degradation and pose significant ecological and human health risks, including mutagenicity and carcinogenicity [2-4]. This hazardous organic complex that enters aquatic and terrestrial ecosystems through anthropogenic activities disrupts habitat physicochemistry and exerts both lethal and sub-lethal toxic effects on resident biota. Meanwhile, persistent challenges faced by oil refineries and petrochemical industries in safely disposing of this recalcitrant waste stream

highlight the urgent need for efficient and sustainable biodegradation strategies to mitigate its long-term environmental and human health impacts. Conventional remediation approaches, such as incineration, chemical oxidation, and stabilisation, have been applied to address crude oil sludge contamination; however, these methods are often cost-prohibitive and generate secondary pollutants, thereby limiting their sustainability, particularly in resource-constrained settings [1, 5-7].

Nevertheless, bioremediation technologies that leverage microbial activity remain a promising approach. The microbial biodegradation offers an environmentally sound and cost-effective remediation pathway by harnessing the metabolic capabilities of indigenous or introduced microorganisms to transform toxic hydrocarbons into inert products [8, 9]. Among the available strategies, tailored co-composting with animal manures has proven uniquely powerful because it simultaneously supplies essential macronutrients nitrogen and phosphorus, introduces phylogenetically diverse microbial inocula, and stimulates ecological succession from mesophilic to thermophilic phases, synergistically enhances PAH solubility and activates co-metabolic degradation pathways [10-12]. In the present study, animal manure amendment emerged as the single most influential ecological driver of bacterial community assembly within crude oil sludge co-compost microcosms, a finding that repositions manure selection from a routine composting variable to a deliberate bioengineering lever for shaping remediation outcomes. Poultry and cow manure treatments supported the highest bacterial richness and functional diversity, selectively enriched taxa that harbour aromatic-ring-cleavage pathways essential for PAH transformation. At the same time, phylogenetic and ecological analyses captured a clear successional trajectory in which generalist heterotrophs that dominated early decomposition phases progressively gave way to specialised hydrocarbon degraders whose metabolic cooperation accelerated the breakdown of structurally complex PAH fractions. This manure-driven succession confers a substantial practical advantage. Within a single composting cycle, the system self-assembles a microbial consortium pre-adapted to petroleum stress, effectively generating a living inoculum that practitioners can harvest for bioaugmentation at sites where indigenous nutrient pools are too depleted to sustain degradation activity independently. The data further reveal a nuanced trade-off between richness and function, whereby cow and poultry manure cultivated the broadest taxonomic diversity, whereas horse and swine/pig amendments channelled community assembly toward fewer but more metabolically potent degraders that achieved superior PAH removal. A pattern that carries a direct design implication, enabling practitioners to select manure type strategically to balance ecological buffering capacity through high diversity for long-term resilience and immediate remediation performance through high specialisation for rapid contaminant reduction. Despite these mechanistic insights, a critical research gap persists in the field. Most existing studies have characterised general composting parameters or targeted pollutant endpoints without resolving the genomic composition and ecological roles of the microbial communities responsible for degradation. A few have examined how distinct manure amendments shape functional specialisation and succession dynamics within hydrocarbon-contaminated systems, thereby limiting the capacity to predict bioremediation outcomes or rationally design bioaugmentation strategies. Directly addressing the provision in a scalable, resource-efficient framework for assembling tailored microbial consortia capable of remediating petroleum-contaminated environments is a contribution of particular significance

for developing regions where locally available animal manures are the most accessible and economically viable route to effective, field-deployable bioremediation.

Recent advances in molecular ecology have transformed our capacity to characterise the microbial communities supporting bioremediation. Amplicon-based profiling of the 16S rRNA gene, combined with next-generation sequencing platforms such as Illumina MiSeq, enables high-level identification of both cultivable and uncultivable taxa, overcoming the limitations of traditional culture-dependent methods [13, 14]. Complementary bioinformatic tools, including refined pipelines such as QIIME2 and Mothur, predictive functional profiling via PICRUSt2, and machine learning-based ecological modelling, now enable deeper insights into microbial interactions and metabolic pathways [15-20]. Studies by Enaregha et al. [21], Kachienga et al. [22], and Hentati et al. [23] have demonstrated vast microbial diversity in oil-contaminated sites, while Sereika et al. [24], Olivier et al. [25], and Mishra et al. [26] introduced long-read amplicon profiling to improve taxonomic resolution. Song et al. [27], Patel et al. [28] and Mukherjee et al. [29] further highlighted global microbial gene sharing in oil reservoirs, and Li et al. [30] and Song et al. [31] linked metagenomic data with metabolomic profiles to elucidate PAH degradation mechanisms.

This study employed amplicon-based metagenomic profiling to characterise dominant bacterial taxa in crude oil sludge co-compost microcosms amended with cow, pig, horse, and poultry manures. Illumina MiSeq-16S rRNA gene sequencing analysis were integrated to elucidate community structure, diversity, and functional potential. Specifically, testing the premise that manure type acts as an ecological filter, differentially selecting for bacterial taxa with distinct hydrocarbon degradation capabilities, thereby shaping both community assembly and bioremediation efficiency.

2. Materials and Methods

2.1 Sample Collection and Characterisation

Crude oil sludge was sourced from a petroleum refinery in Durban, South Africa, and characterised using automated Soxhlet extraction with dichloromethane followed by gas chromatography–mass spectrometry (GC/MS) to quantify PAH content. Animal manures, cow, swine/pig, horse, and poultry were obtained from the University of Pretoria Farm, South Africa, and analysed for total organic carbon (TOC), nitrogen (N), and phosphorus (P) using standard methods [32, 33]. Garden soil (20cm depth) collected from a farm in Tembisa (north of Kempton Park, East Rand, Gauteng, South Africa; S 26°1'0", E 28°14'0") was homogenised and characterised for texture, pH, water holding capacity (WHC), and elemental composition using inductively coupled plasma optical emission spectrometry (ICP-OES PerkinElmer Optima 5300 DV) using concentrated acid digestion by a concentrated aqua regia protocol (HNO₃:HCl, 1:3, v/v) and analysed for multi element nutrients/metals with a 23 element Merck stock (cat no: 43843 standard stock solution; 100 mg L⁻¹) used to prepare ten calibration levels (0.2–8 mg L⁻¹; ppm = parts per million and mg/L = milligram per litre); working standards were prepared in nitric acid and filtrates from digested soil sample were quantified under identical conditions [33, 34].

2.2 Co-Compost Microcosm Setup

Microcosms were prepared by mixing 300 g of crude oil sludge with 400 mL of petroleum ether (C₆H₁₄, 95%) to dissolve the crude oil sludge prior to mixing with 1 kg of soil; the mixture was air-dried. After air-drying to remove residual solvent, wood chips were added in a 1:2 w/v

ratio (soil: wood chips), followed by incorporation of 1.17 kg of individual manures in a 2:1 w/w ratio. A control treatment without manure was included. Each treatment was replicated three times and incubated in aerated PVC troughs (30 × 18 × 20 cm) at room temperature for 300 days. Moisture levels were maintained between 50% and 80%, and pH was monitored monthly. Compost maturity and microbial activity were evaluated using a modified closed-jar method assessing CO₂ evolution [35, 36]. Ash content was evaluated at the beginning and at the end of the experiment. All reagents used in this study were of analytical grade and were used without further purification. Composting treatments were designed based on laboratory conditions, which were maintained at 22 °C and 100 kPa.

2.3 Culture-Dependent Isolation of Hydrocarbon-Degrading Bacteria

To enrich hydrocarbon-degrading bacteria, 15 g of compost samples were inoculated into 100 mL of mineral salts medium (MSM) supplemented with 10 mL of crude oil sludge as the sole carbon source. Cultures were incubated at 28°C for 21 days under agitation, followed by subculturing to enrich adapted strains. Serial dilutions (10⁻⁶ to 10⁻⁸) were plated on mineral salts agar (MSA), and distinct colonies were purified on nutrient agar. Isolates were subsequently cultured in nutrient broth for DNA extraction using the Quick g-DNA™ kit (Zymo Research, USA) according to the manufacturer's protocol and for further analysis.

2.4 DNA Extraction, Sequencing, and Bioinformatic Analysis: Quality Assessment

To unravel the microbial dynamics and biodegradation potential within crude oil sludge co-compost microcosms, a robust and meticulously optimised workflow was used for DNA extraction, amplification, sequencing, and bioinformatics analysis. For compost samples, 15 g was suspended in 50 mL phosphate-buffered saline, incubated overnight, homogenised, and centrifuged at 12,000 rpm for 5 minutes at 4°C. Total DNA was extracted from the supernatant using the Faecal/Soil Total DNA™ kit (Zymo Research Corporation) according to the manufacturer's protocol, to ensure comprehensive recovery of microbial DNA from complex matrices. Total community genomic DNA extracted from both pure bacterial isolates and compost samples was pooled for downstream analysis. DNA integrity was confirmed via 1% agarose gel electrophoresis (Supplementary File 1), and quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Thus, samples that exhibited optimal purity ratios (A₂₆₀/A₂₈₀ = 1.8–2.0) and concentrations of 20–150 ng/μL were used for downstream applications.

2.5 PCR Amplification, Library Preparation and Sequencing

PCR amplification of the 16S rRNA gene was performed using a two-step strategy. Initial amplification employed universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') spanning full-length variable regions. A nested PCR then amplified the V1–V3 hypervariable regions using primers 27F and 518R (5'-GTATTACCGCGGCTGCTGG-3'), selected for their high taxonomic resolution. PCR conditions comprised: initial denaturation at 95°C for 10 min; 32 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min); and a final extension at 72°C for 10 min. Amplicons were purified using the Zymo DNA Clean & Concentrator™ kit.

Libraries were prepared using adapter sequences compatible with Illumina indexing [38] and sequenced using paired-end 300 bp reads with v3 chemistry on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the University of South Africa, Florida Campus.

2.6 Bioinformatic Analysis

Raw sequencing reads were subjected to quality control using ngsShoRT [39], which trimmed low-quality bases, removed ambiguous nucleotides, and filtered PCR artefacts. Chimeric sequences were identified and eliminated using the UCHIME algorithm [39, 40]. High-quality reads were processed through the Mothur pipeline (v1.40.0) [42], aligned against the SILVA 16S rRNA database (releases 128 and 132) [43], and clustered into operational taxonomic units (OTUs) at 97% sequence similarity. Taxonomic classification used the Naïve Bayesian classifier with an 80% confidence threshold [44], and representative OTUs were validated against the NCBI nucleotide database using BLAST [45].

Alpha diversity metrics, including Shannon–Weaver and Chao1 indices, were calculated at a genetic distance of 0.03, with Good’s coverage estimator used to assess sequencing depth. Beta diversity was explored using Bray–Curtis dissimilarity, non-metric multidimensional scaling (NMDS), and principal component analysis (PCA) via the vegan and phyloseq packages [45, 46]. Canonical Correspondence Analysis (CCA) was applied to correlate microbial abundance with PAH reduction levels. Differential abundance analysis was conducted using LEfSe [48] and DESeq2.

2.7 Functional Validation

The metabolic competence of enriched bacterial consortia was validated through complementary assays. PCR screening for the catechol 2,3-dioxygenase (*cbzE*) gene, which encodes a key enzyme in aromatic ring cleavage, confirmed the genetic capacity for PAH degradation. A heatmap visualisation of PAH-degrading genera with crude oil sludge was generated to assess substrate-specific patterns. Functional profiling was performed using PICRUSt2 [48, 49, 50], with Nearest Sequenced Taxon Index (NSTI) scores validating prediction accuracy. KEGG Orthology (KO) assignments inferred metabolic pathways involved in hydrocarbon degradation.

2.8 PAH Quantification

PAHs were extracted from compost samples using automated Soxhlet extraction with dichloromethane via EPA Method 3541 [51, 52] and quantified by GC/MS (Agilent 7860GC, 5975C MSD) following EPA Method 8270. Calibration standards (10–50 ppm) were prepared from a 1,000-ppm stock standard (Restek Cat No. 8270-1). Chromatographic conditions included helium as carrier gas (1 mL/min), splitless injection (1 µL), and a temperature gradient from 40°C to 310°C. Recovery rates were validated against the standards [53, 54].

2.9 Statistical Analysis

Community composition was visualised using stacked bar charts and heatmaps generated with ggplot2 [53] and heatmap. 2 in R (v3.6.1) [57, 58]. PERMANOVA was applied to test for significant differences in community composition across treatments. Community composition was analysed using a suite of multivariate statistical tests. Beta diversity was quantified using the Bray–Curtis dissimilarity index computed from genus-level relative abundance profiles. Non-metric multidimensional scaling (NMDS) was performed to visualise community compositional differences among treatments in reduced ordination space, with a stress value < 0.05 indicating excellent two-dimensional representation.

Permutational multivariate analysis of variance (PERMANOVA) [56] was conducted using the adonis2-equivalent algorithm with 9,999 unrestricted permutations of raw data on the Bray–Curtis dissimilarity matrix to test for significant differences in bacterial community composition across the five co-compost treatments (Poultry Manure, Horse Manure, Cow

Manure, Swine/Pig Manure, and unamended Control). Pairwise PERMANOVA comparisons between all treatment pairs were corrected for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. Analysis of similarities (ANOSIM) [57] was performed as a complementary rank-based test. Homogeneity of multivariate dispersions was assessed using the PERMDISP procedure [58] to verify the PERMANOVA assumption of equal group dispersions. Similarity percentage analysis (SIMPER) [57] was used to identify the genera that contributed most to the between-treatment dissimilarity. The Mantel test (9,999 permutations) assessed the correlation between community compositional dissimilarity and PAH degradation profiles. Environmental vector fitting projected PAH degradation variables (LMW reduction, HMW reduction, mean PAH reduction, mean residual concentration) onto the NMDS ordination space to visualise relationships between community structure and bioremediation outcomes. Alpha diversity indices (Shannon H', Simpson 1-D, Chao-1, Fisher's alpha, Evenness) were calculated from OTU-level data.

3. Results

3.1 Characterisation and Changes in physicochemical parameters during co-composting of crude oil sludge and animal manures

Co-composting of crude oil sludge with poultry, cow, horse, and pig manures produced clear, treatment-dependent shifts in temperature, moisture, pH, and respiration, indicating that manure amendment substantially altered the physicochemical system of the microcosms. The soil used was a sandy loam with mildly acidic pH (5.56), appreciable organic carbon (13.01 mg kg⁻¹), and moderate water-holding capacity (32.62 % WHC), providing a permissive base matrix for microbial activity. The crude oil sludge itself contained a complex mixture of low- and high-molecular-weight PAHs, including naphthalene, anthracene, pyrene, chrysene, benzo[a]pyrene, indeno(1,2,3-cd)pyrene and more, confirming that the compost systems were challenged with chemically diverse and environmentally relevant hydrocarbon substrates.

Temperature increased in all manure-amended piles but remained comparatively low and stable in the unamended control. Poultry manure produced the highest thermal response, rising from 22.5 °C to 27.3 °C in the first month, while pig and cow treatments reached maxima of 25.7 °C, and the horse treatment peaked at 24.2 °C. In contrast, the control remained close to ambient temperature at approximately 22–23 °C throughout the experiment. Moisture content also rose markedly in amended treatments, generally exceeding 50% during active composting, with poultry, pig, and horse treatments reaching sustained high values, whereas the control remained below 46% for most of the period. Cow manure showed a slower moisture build-up early in the process, consistent with delayed warming. pH increased from acidic initial values toward neutral to alkaline conditions in all amended piles, with poultry reaching the highest pH of 7.9, and the other manure treatments also rising into ranges favourable for bacterial activity, 5.3±0.1 to 7.8±0.1, before later fluctuations. By comparison, the control showed only a modest pH increase and remained less dynamic overall. Ash content changed very little between the beginning and end of composting in all treatments, indicating that mineral composition remained essentially stable during the process.

The strongest evidence of biologically active composting came from the respiration profiles. CO₂ evolution increased in every manure-amended treatment over time, with poultry manure showing the greatest respiratory response, rising from 4.6 ± 0.01 to 18.7 ± 0.12, followed by

horse manure from 2.3 ± 0.1 to 12.9 ± 0.1 – 14.2 ± 0.1 , pig manure from 2.3 ± 1.0 to 12.7 ± 0.1 , and cow manure from 2.1 ± 0.1 to 11.1 ± 0.1 . The control showed only a limited increase, reaching 6.5 ± 0.1 before declining again.

The four animal manures used as co-composting amendments differed sharply in their organic carbon and phosphorus loadings, while total organic nitrogen remained comparatively uniform across all sources. Total organic carbon (TOC) spanned an elevenfold range, rising from the lowest concentration in horse manure ($81 \pm 3 \text{ mg kg}^{-1}$) and cow manure ($109 \pm 8 \text{ mg kg}^{-1}$), through an intermediate value in poultry manure ($277 \pm 63 \text{ mg kg}^{-1}$), to a commanding maximum in pig manure ($904 \pm 84 \text{ mg kg}^{-1}$). Pig manure thus exceeded poultry manure by more than threefold, cow manure by roughly eightfold, and horse manure by over elevenfold, establishing a steep and unambiguous carbon gradient across the four amendments.

Total organic nitrogen (TON) behaved very differently, holding remarkably steady across manure types within a narrow band of only 5.7 mg kg^{-1} . Poultry manure recorded the lowest value at $49.2 \pm 14.2 \text{ mg kg}^{-1}$, followed in ascending order by pig manure ($50.6 \pm 5.9 \text{ mg kg}^{-1}$), horse manure ($52.7 \pm 2.7 \text{ mg kg}^{-1}$), and cow manure ($54.9 \pm 5.9 \text{ mg kg}^{-1}$). The coefficient of variation across the four nitrogen measurements remained below 12%, confirming that all four amendments delivered a broadly equivalent nitrogen input to the crude oil sludge matrix.

Total organic phosphorus (TOP) tracked the carbon gradient rather than the nitrogen background and partitioned the four manures into two clearly demarcated nutrient tiers. Poultry manure ($254 \pm 14 \text{ mg kg}^{-1}$) and pig manure ($252 \pm 29 \text{ mg kg}^{-1}$) together formed a high-phosphorus tier, delivering concentrations approximately five times greater than those measured in horse manure ($50 \pm 2 \text{ mg kg}^{-1}$) and cow manure ($46 \pm 8 \text{ mg kg}^{-1}$), which together constituted a corresponding low-phosphorus tier. This sharp phosphorus dichotomy, superimposed on the TOC gradient and the uniform TON baseline, generated two fundamentally distinct nutrient regimes across the experiment: a high-C / high-P regime supplied by pig and poultry manures, and a low-C / low-P regime supplied by cow and horse manures, with organic nitrogen held approximately constant across both. This natural factorial architecture, in which carbon and phosphorus covary by five- to elevenfold while nitrogen remains essentially fixed, provided a quasi-controlled stoichiometric framework for the subsequent microbial and bioremediation analyses. These physicochemical differences coincided with measurable reductions in selected PAHs across all manure-amended microcosms relative to the unamended control, indicating that co-composting with each of the four manures established conditions broadly conducive to hydrocarbon biodegradation, albeit to varying degrees across treatments.

3.2 Sequencing Output and Taxonomic Overview

3.2.1 Multi-Dimensional Alpha Diversity Profiling of Bacterial Communities Across Co-Compost Treatments

Amplicon-based sequencing of the 16S rRNA gene (Illumina MiSeq, V1–V3 hypervariable regions) recovered a total of 125,972 high-quality reads after stringent quality filtering and chimera removal through the Mothur pipeline (v1.40.0), aligned against the SILVA database (releases 128/132). These reads resolved into 359 genera spanning 17 phyla, 42 classes, 83 orders, and 162 families at 97% sequence similarity. Good's coverage exceeded 98% across all five treatments and reached 99.7% in poultry manure (PM), confirming near-complete

capture of resident bacterial diversity (Table 1). The community structure was characterised using thirteen complementary indices across four independent ecological dimensions, richness, evenness, dominance, and rare-taxon sensitivity, to provide a level of analytical resolution that permits metric selection.

Taxonomic richness varied nearly fourfold across treatments with diversity or functional performance. Cow manure (COW) harboured the greatest number of observed OTUs (422), corroborated by five convergent richness estimators: Chao-1 (422.6), Margalef (43.67), Menhinick (3.403), and Fisher's α (80.21), the latter indicating an exceptionally long tail of rare taxa. Despite this richness, COW displayed only moderate Shannon diversity ($H' = 3.726$) and low evenness ($e^H/S = 0.098$), indicating that the high total organic carbon conditions (TOC = 109 mg/L) sustained a broad but unequally distributed generalist assemblage. Poultry manure ranked second in OTU count (309) yet dominated every diversity and evenness metric: it recorded the highest Shannon entropy ($H' = 4.065$), highest Simpson diversity ($1-D = 0.954$), lowest dominance ($D = 0.046$), lowest Berger–Parker index (0.144), and the highest Equitability ($J = 0.709$) and Brillouin (4.048) values, collectively indicating a community in which no single taxon monopolised the assemblage. Horse manure occupied an intermediate ecological position with 212 OTUs, a Shannon index of 2.814, and moderate dominance ($D = 0.121$; Berger–Parker = 0.238) (Figure 1), consistent with its lower nitrogen content (52.7 mg/L), providing neither the strong selective pressure of pig manure nor the broad nutrient base of cow manure.

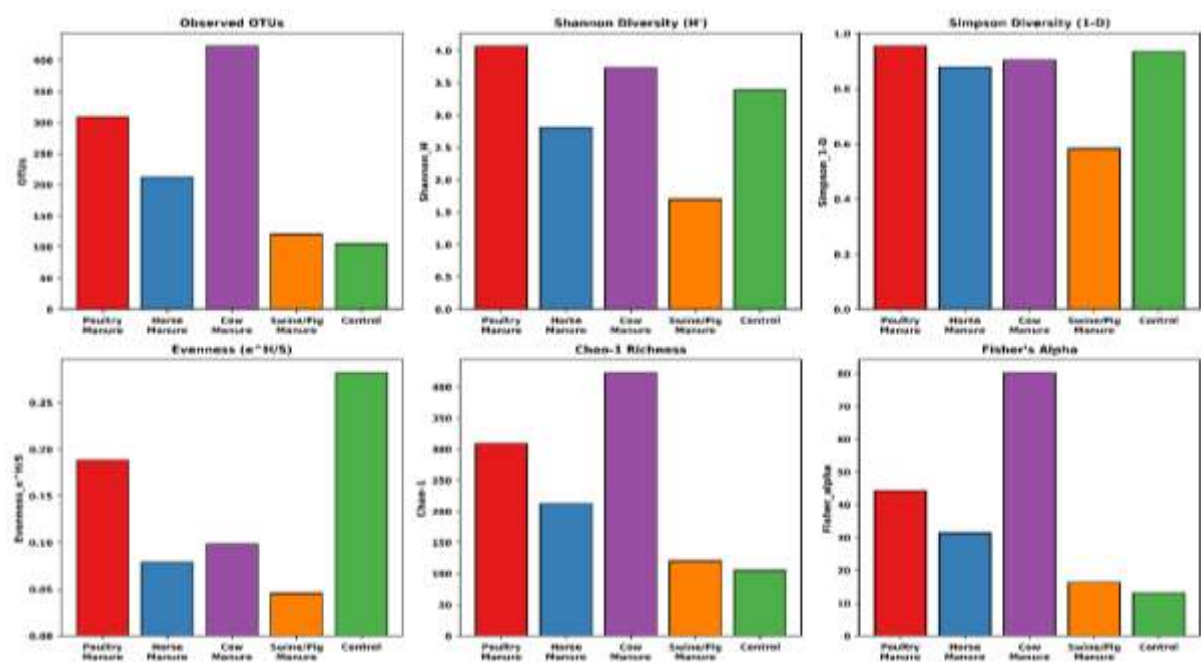


Figure 1. Alpha diversity indices across the co-compost treatments.

The most striking ecological signature belonged to swine/pig manure (SPM). Receiving the highest carbon (904 mg/L) and phosphorus (252 mg/L) inputs of any treatment, SPM supported only 120 OTUs and recorded the lowest Shannon diversity ($H' = 1.698$), the highest dominance ($D = 0.417$), and a Berger–Parker index of 0.631, meaning a single taxon captured over 63% of all sequence reads. Its evenness ($e^H/S = 0.046$) and Equitability ($J = 0.355$) were the lowest

across all treatments. The unamended control (CT) supported the fewest OTUs (105) yet maintained the highest evenness of any treatment ($e^{H/S} = 0.282$; $J = 0.728$) and moderate Shannon diversity ($H' = 3.388$), indicating a stable, equitably distributed indigenous community in the absence of exogenous nutrient inputs.

When treatments are ranked by taxonomic richness, COW (422) > PM (309) > HM (212) > SPM (120) > CT (105), the order is effectively reversed when ranked by PAH degradation efficiency, with SPM and PM achieving up to 99.9% removal of low-molecular-weight PAHs despite possessing fewer OTUs than the less effective COW and CT treatments (Table 1).

Table 1. Summary of sequencing output and ecological diversity indices for bacterial communities in crude oil sludge co-compost microcosms amended with different animal manures.

Diversity Metric	Animal Manure-Amended Co-Compost Treatments				
	HM ^a	SPM ^b	COW ^c	PM ^d	CT ^e
Total reads	26,884	25,972	15,377	47,975	40,129
Observed OTUs	212	120	422	309	105
Chao1 richness	212.00	120.40	422.60	309.00	105.00
Shannon (H')	2.814	1.698	3.726	4.065	3.388
Simpson ($1-D$)	0.879	0.583	0.904	0.954	0.933
Dominance (D)	0.121	0.417	0.096	0.046	0.067
Evenness ($e^{H/S}$)	0.079	0.046	0.098	0.189	0.282
Equitability (J)	0.525	0.355	0.616	0.709	0.728
Brillouin	2.798	1.688	3.675	4.048	3.380
Menhinick	1.293	0.745	3.403	1.411	0.524
Margalef	20.690	11.710	43.670	28.580	9.811
Fisher α	31.390	16.270	80.210	44.200	13.080
Berger-Parker	0.238	0.631	0.278	0.144	0.189
Goods coverage (%)	99.7	98.5	99.2	98.6	99.0

^a HM = horse manure-amended compost treatment; ^b SPM = swine/pig manure-amended compost treatment; ^c COW = cow/dairy manure-amended compost treatment; ^d PM = poultry/layer manure-amended compost treatment; ^e CT = unamended control (crude oil sludge + soil + wood chips, no manure addition).

Index: Shannon (H') = Shannon–Weaver entropy index quantifying community diversity; Simpson ($1-D$) = probability that two randomly drawn individuals belong to different OTUs; Dominance (D) = complement of Simpson ($1-D$), reflecting community unevenness; Evenness ($e^{H/S}$) = exponential Shannon divided by richness; Equitability (J) = Pielou’s evenness index ($H'/\ln S$); Brillouin = diversity index appropriate for fully censused collections; Menhinick = richness estimator ($S/\sqrt{N}$); Margalef = richness estimator ($(S-1)/\ln N$); Fisher α = log-series alpha sensitive to rare taxa; Berger–Parker = proportional abundance of the single most dominant taxon; Chao1 = non-parametric richness estimator accounting for unseen species. OTU = operational taxonomic unit (97% similarity); PAH = polycyclic aromatic hydrocarbon.

Rarefaction analysis confirmed that sequencing depth was sufficient to capture the vast majority of detectable bacterial diversity across all five co-compost treatments (Figure 2). Every curve approached a clear asymptote, and Good’s coverage exceeded 98% in all samples, reaching 99.7% in poultry manure, establishing that observed differences in OTU richness reflect genuine biological variation rather than artefacts of unequal sampling effort. Rarefaction

curves plateaued across all treatments (Figure 1), indicating saturation in OTU discovery and validating sequencing depth. By contrast, swine/pig manure (SPM) and the unamended control (CT) plateaued early and lower, even though SPM received nearly three times the sequencing depth of COW. The inability of additional sequencing effort to uncover substantially more taxa in these treatments confirmed that their communities are genuinely depauperate rather than under-sampled, a critical distinction for downstream ecological inference. The vertical separation between the highest curve (COW, 422 OTUs) and the lowest (CT, 105 OTUs) spanned a fourfold range, quantifying the magnitude of manure-driven divergence in community richness across treatments that shared an identical crude oil sludge substrate.

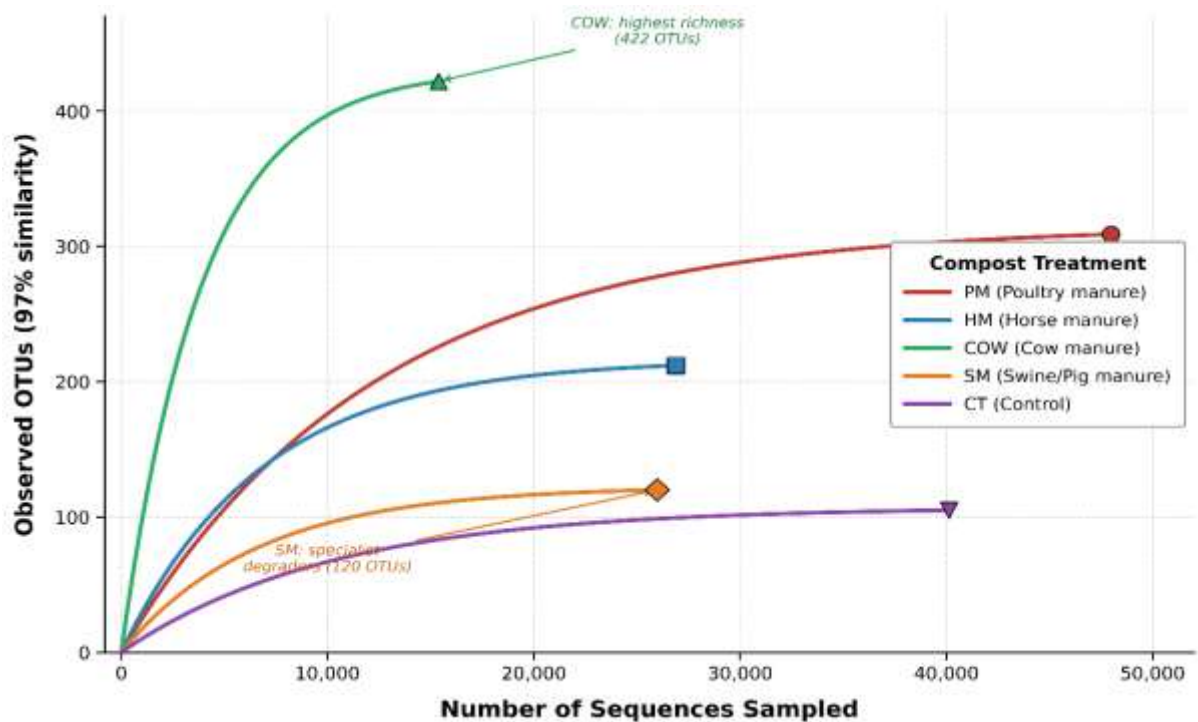


Figure 2. Rarefaction curves indicating the observed number of operational taxonomic units (OTUs) within the 16S rRNA gene sequences of crude oil sludge samples composted using different animal manures. OTUs are defined at the 3% genetic distance threshold (97% sequence similarity). Treatments: PM = poultry manure; HM = horse manure; COW = cow/dairy manure; SM = swine/pig manure; CT = unamended control. Endpoint markers (●, ■, ▲, ◆, ▼) indicate final observed OTU counts at total sequencing depth. Curve plateauing confirms sufficient sampling coverage across all treatments (Good's coverage > 98%).

3.3 Manure-Driven Shifts in Bacterial Community Structure

Amplicon-based profiling of four co-composting microcosms and a control yielded a high-quality read set distributed across operational taxonomic units (OTUs) after quality filtering, chimera removal, and taxonomic classification against the Genome Taxonomy Database (GTDB) reference database (Figure 1 and Table 1). The asymmetry in sequencing depth reflects both the differential microbial biomass recovered from each treatment and the inherent stochasticity of library preparation, highlighting the importance of rarefaction-aware normalisation in downstream beta diversity analyses (Figure 2).

3.3.1 Phylum-Level Community Architecture Reveals Divergent Assembly Trajectories

Taxonomic assignment resolved sequences into the top 17 bacterial phyla, with six phyla, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Acidobacteria*, *TM6*, and others, collectively accounting for over 95% of total classified reads. Notably, the dominance architecture differed profoundly between manure-amended and control-unamended microcosms, providing the first indication that organic amendment identity exerts a deterministic filter on bacterial community assembly during crude oil sludge co-composting. Taxonomic classification of 16S rRNA gene sequences across the treatments revealed a hierarchical pattern of manure-driven community divergence that operated simultaneously at the phylum, class, and order levels (Figure 3).

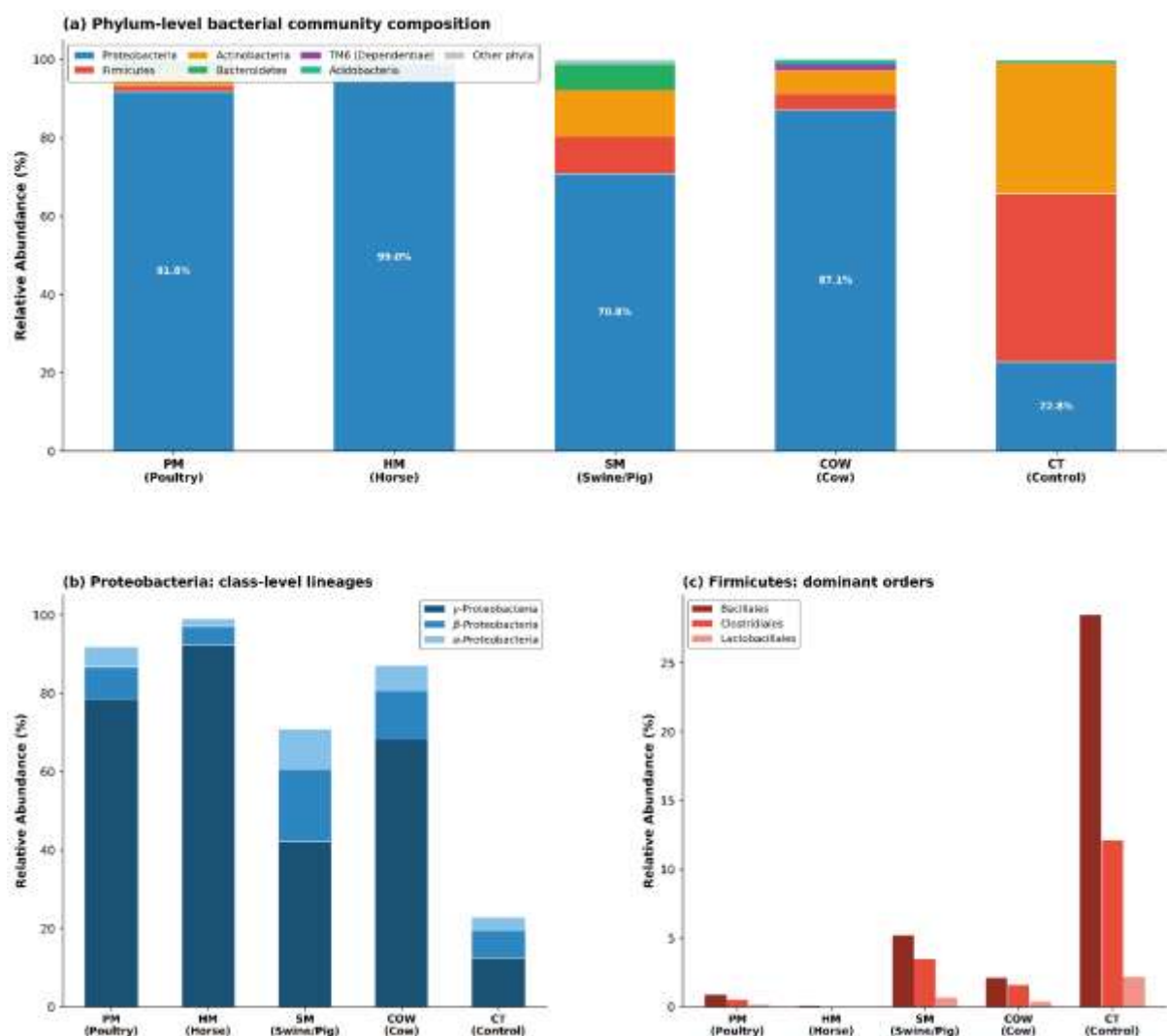


Figure 3. Comparative Taxonomic Distribution of Bacterial Communities Across Co-Compost Treatments with poultry (PM), horse (HM), cow (CM), and swine (SM) manures and control samples (CT): Multi-Level Profiling Reveals Manure-Specific Community Architectures. Diversity at the phylum level (a) and *Proteobacteria* into α -, β -, and γ -class level lineages is shown in (b). The *Firmicutes* dominant orders in the phylum (c).

At the phylum level, *Proteobacteria* overwhelmingly dominated four of the five treatments, accounting for 99.0% of all classified reads in horse manure (HM), 91.8% in poultry manure (PM), 87.1% in cow manure (COW), and 70.8% in swine/pig manure (SM) (Figure 3a). This near-monopoly declined to 22.8% in the unamended control (CT), where the community

exhibited extensive compositional reorganisation, with *Firmicutes* (42.8%) and *Actinobacteria* (33.4%) emerging as co-dominant taxa and together accounting for over 76% of the total abundance. Swine/pig manure occupied a uniquely intermediate position, although *Proteobacteria* still prevailed. SPM supported the most phylogenetically pluralistic structure among the manure-amended treatments, with meaningful contributions from *Actinobacteria* (11.9%), *Firmicutes* (9.4%), and *Bacteroidetes* (6.4%). Cow manure also diverged from the HM and PM pattern by retaining a notable *Actinobacteria* component alongside its *Proteobacterial* majority.

Resolving *Proteobacteria* into α -, β -, and γ -class lineages revealed an additional layer of ecological selection that was not apparent at the phylum level (Figure 3b). γ -*Proteobacteria* dominated overwhelmingly in HM and PM, consistent with the selective enrichment of *Pseudomonas* and related genera that carry well-characterised naphthalene dioxygenase and catechol dioxygenase pathways. In SM, however, the class distribution shifted toward a more balanced representation of γ -, β -, and α -*Proteobacteria*. The elevated contribution of β -*Proteobacteria* in SM was particularly striking, given that this class includes *Burkholderia*–*Paraburkholderia*, genera with broad-substrate oxygenase systems that support co-metabolic transformation of structurally complex PAHs. COW retained a substantial α -*Proteobacteria* fraction, consistent with its enrichment of oligotrophic lineages such as *Bradyrhizobium*, *Methylobacterium*, and *Sphingomonas*. The control treatment contributed only minimal *Proteobacteria* across all three classes, confirming that without manure-driven nutrient input, the ecological conditions did not favour the establishment of any *Proteobacterial* degrader lineage in substantial abundance.

The *Firmicutes* order-level breakdown resolved why the control's high overall evenness (Equitability $J = 0.728$) failed to translate into effective bioremediation (Figure 3c). *Bacillales* (~19%) and *Clostridiales* (~12%) dominated CT's *Firmicutes* fraction, together contributing over 30% of total community abundance. SM showed moderately elevated *Bacillales* (5.2%) and *Clostridiales* (3.5%), whereas PM and HM carried these orders at trace levels (< 1%). Cow manure maintained intermediate *Firmicutes* representation (*Bacillales* ~2.5%; *Clostridiales* ~2.0%), consistent with its broader taxonomic architecture.

3.3.2 Cross-Treatment Phylum Connectivity Resolved by Circos Analysis

The Circos chord diagram visualised the connectivity between all the treatments and the bacterial phyla detected across the combined dataset, with ribbon width scaled proportionally to relative abundance and outer arc length reflecting each taxon's cumulative contribution to the total community (Figure 4).

Proteobacteria dominated the phylum-level architecture, accounting for 74.3% of all classified sequences across the combined dataset and sending broad ribbons to every treatment. However, the width of these ribbons varied conspicuously: the thickest *Proteobacteria* chords connected to horse manure (HM) and poultry manure (PM), consistent with the 99.0% and 91.8% treatment-level *Proteobacterial* abundances reported in the stacked bar analysis, while progressively thinner ribbons extended toward swine/pig manure (SPM), cow manure (CM), and the unamended control (CT). This asymmetric ribbon architecture confirmed that, although *Proteobacteria* were ubiquitous, their quantitative dominance was concentrated overwhelmingly in the herbivore and avian manure treatments.

Firmicutes (11.6%) and *Actinobacteria* (11.0%) together accounted for nearly all remaining community membership. The *Firmicutes* arc sent its thickest ribbon to the control treatment, with a secondary connection to cow manure and a moderate chord to swine manure, directly reflecting the *Bacillales*- and *Clostridiales*-dominated assemblage documented in CT. The *Actinobacteria* are similarly directed, with their widest ribbons toward the control and cow manure treatments, consistent with the enrichment of slow-growing *actinobacterial* lineages (*Gordonia*, *Dietzia*, *Rhodococcus*) under the lignocellulosic and humic substrate conditions these microcosms provided. *Bacteroidetes* contributed 2.1% of total sequences, with its most visible chord connecting to swine manure, the treatment with the most phylogenetically diverse profile. The candidate phylum *TM6* (*Dependentiae*; 0.3%), *Acidobacteria* (0.4%), and other minor phyla (0.3%) contributed narrow ribbons distributed sparsely across treatments, with *Acidobacteria* connecting primarily to cow manure. The equal outer arc allocation to each treatment (20.0%), reflecting the balanced study design, permitted direct visual comparison of ribbon thickness across treatments without the confounding influence of unequal sample sizes.

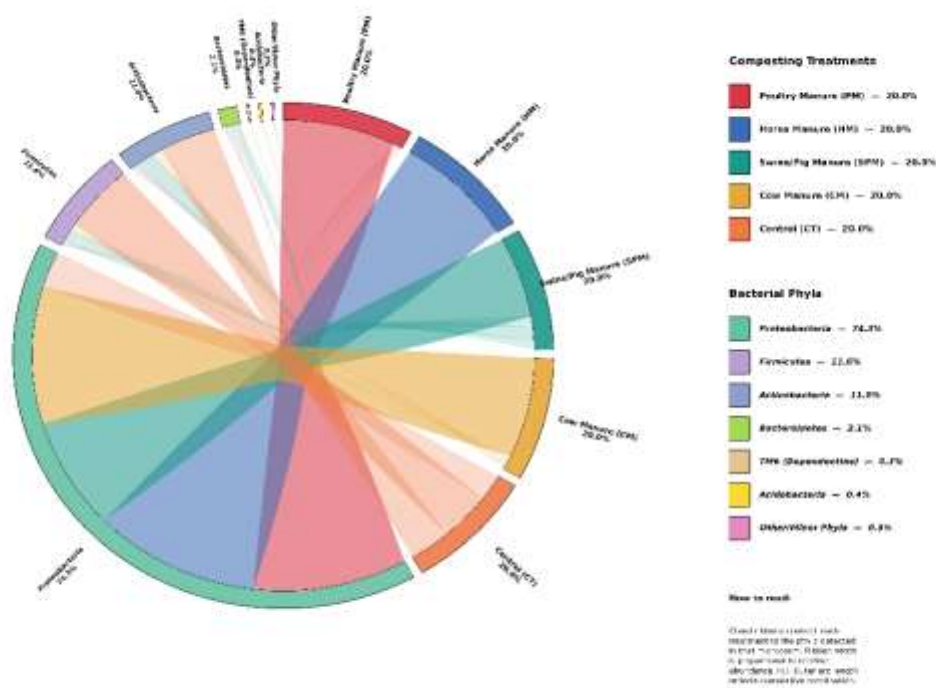


Figure 4. Circos chord diagram illustrating the phylogenetic connectivity between the crude oil sludge co-compost treatments and the bacterial phyla detected across the combined 16S rRNA gene amplicon dataset. The right semicircle displays the composting treatments (PM, Poultry Manure; HM, Horse Manure; SPM, Swine/Pig Manure; CM, Cow Manure; CT, Control), each allocated equal arc length (20.0%) reflecting the balanced experimental design. The left semicircle displays bacterial phyla, with arc length proportional to cumulative relative abundance across all treatments: *Proteobacteria* (74.3%), *Firmicutes* (11.6%), *Actinobacteria* (11.0%), *Bacteroidetes* (2.1%), *Acidobacteria* (0.4%), *TM6/Dependentiae* (0.3%), and other minor phyla (0.3%). Chord ribbons connect each treatment to the phyla detected in that microcosm, with ribbon width scaled proportionally to relative abundance (%). The asymmetric distribution of *Proteobacteria* ribbons, thickest to HM and PM, thinnest to CT, visualises the manure-driven ecological filtering mechanism that restructures phylum-level community composition across treatments.

3.4 Genus-Level Community Structure and Core Microbiome Overlap Across Co-Compost Treatments and Specific Partitioning of Dominant Assemblage

Heatmap visualisation of $\log_{10}$ -transformed relative abundance for the top 40 genera revealed sharp treatment-specific partitioning of bacterial taxa across the five microcosms (Figure 5a). *Pseudomonas* emerged as the single most abundant genus in the entire dataset, yet its distribution was profoundly uneven. It dominated horse manure (HM, 70.2%) and poultry manure (PM, 47.0%), contributed substantially to swine/pig manure (SPM, 17.1%) and cow manure (CM, 12.7%), but was supplanted in the control (CT) by an entirely different taxonomic assemblage. The heatmap colour gradient visually captured this transition, intense red (high abundance) concentrated among *Proteobacterial* genera on the left side of the matrix for manure-amended treatments, shifting to deep blue (low abundance) across the same genera in CT, which instead displayed its own red-dominant block centred on *Bacillus* (28.4%), *KCM-B-112* (17.4%), *Dietzia* (11.3%), and *Nocardiopsis* (7.6%).

Each manure treatment assembled a taxonomically distinctive degrader guild beyond the shared *Pseudomonas* signal. Poultry manure co-enriched *Rhodanobacter* (22.6%) and *Achromobacter* (10.7%), with *Pseudoxanthomonas* reaching 4.6%. Horse manure showed the most extreme single-genus dominance, with *Pseudomonas* alone accounting for over 70% of all reads, supplemented by *Achromobacter* (12.0%), *Stenotrophomonas* (4.5%), and *Microbacterium* (3.3%). Swine/pig manure recruited a more distributed profile, *Enterobacter* (13.2%), *Bacillus* (7.8%), *Sedimentibacter* (13.2%), *Dietzia* (5.1%), *Citrobacter* (3.9%), and *Clostridium sensu stricto* (6.6%), consistent with the phylogenetically diverse phylum-level architecture documented for this treatment. Cow manure harboured the most taxonomically distinct profile of any manure-amended treatment: *Proteiniphilum* (20.3%), *Delftia* (5.5%), *Dietzia* (10.4%), *Propionibacterium* (5.3%), and *Gordonia* (3.3%) collectively dominated a community in which no single genus achieved the overwhelming monopoly observed in HM or PM.

Venn analysis across all five treatments detected 316 genera in total, of which only 13 were shared universally, representing a conserved core microbiome that persisted regardless of manure input (Figure 5b). Treatment-specific richness varied markedly: CT harboured 179 genera (76 unique to CT alone), SPM supported 159 (44 unique), PM contained 119 (27 unique), CM contained 72 (15 unique), and HM supported 69 (18 unique). The control's disproportionately high unique genus count (76) reflected its fundamentally different community architecture, dominated by *Firmicutes* and *Actinobacteria*.

When the control was excluded, the manure core microbiome expanded marginally to 14 shared genera, indicating that one additional genus was consistently recruited by manure amendment but absent from unamended compost (Figure 5c). Pairwise overlaps revealed that PM and SPM shared the largest exclusive genus pool (43 genera), representing the strongest inter-treatment compositional convergence in the dataset. SPM and CM shared 9 exclusive genera, PM and HM shared 5, and HM and CM shared 7. Treatment-specific unique genera were highest for SPM (67) and PM (36), followed by HM and CM (20 each), confirming that swine and poultry manures imposed the strongest selective pressures for treatment-exclusive taxa.

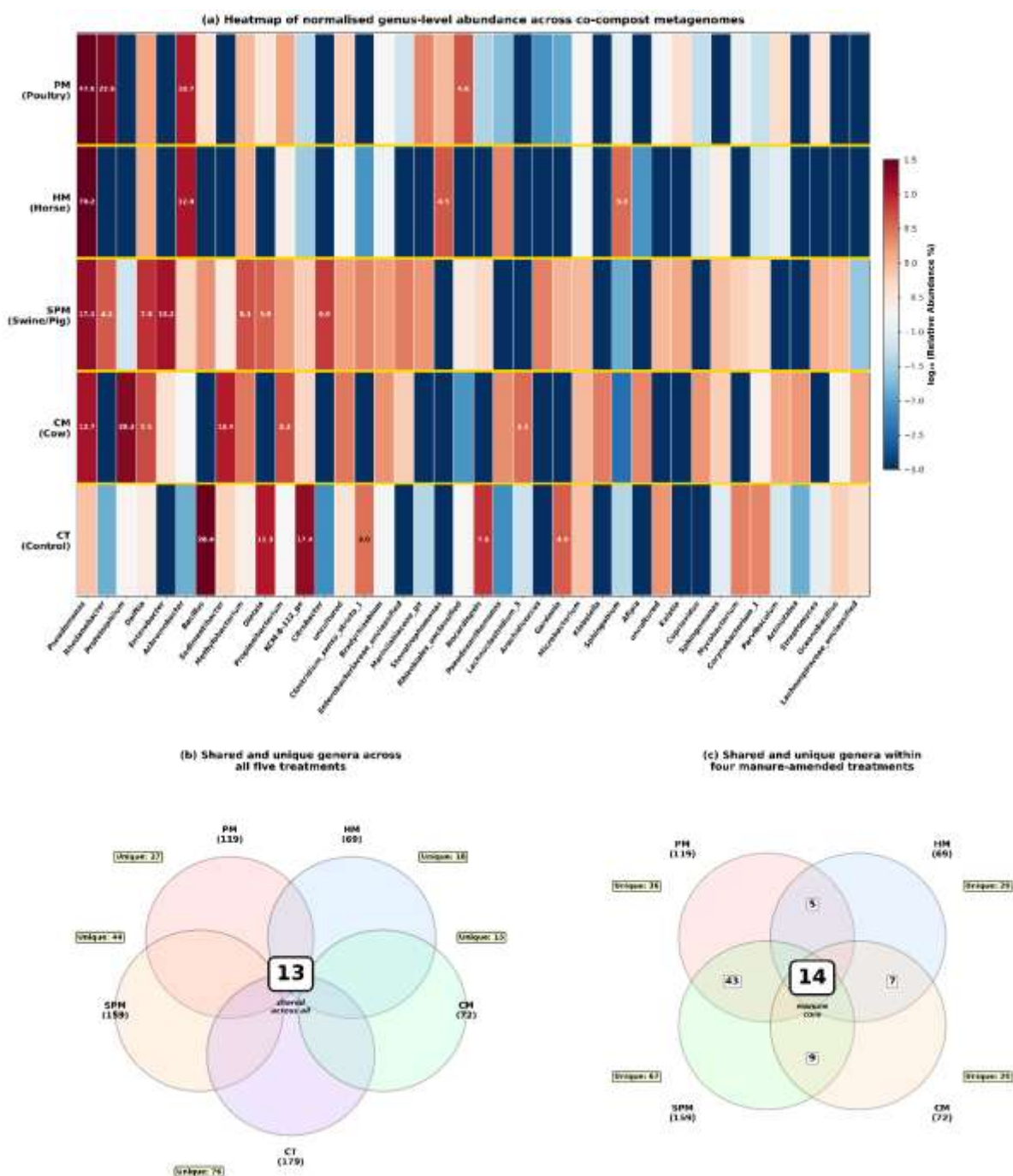


Figure 5. Relative taxonomic distribution of genera and common microbiome diversity in crude oil sludge co-compost treatments. (a) Heatmap of normalised abundance at the genus level for bacteria across five treatment metagenomes: PM (poultry), HM (horse), SPM (swine/pig), CM (cow), and CT (control). Colour scale represents $\log_{10}$ -transformed relative abundance (%), with red indicating higher and blue indicating lower abundance. Values $>3\%$ are annotated directly on cells. The top 40 genera ranked by total read count are displayed. (b) Venn diagram showing shared and unique genera across all five treatments. A total of 316 genera were detected, with 13 shared across all treatments and 71 unique to the control. (c) Venn diagram showing shared and unique genera within the four manure-amended treatments only. A core of 14 genera was shared across all manure treatments, with pairwise overlaps indicating treatment-specific recruitment patterns.

3.5 Beta Diversity Analysis: Bray–Curtis Dissimilarity Reveals Fundamental Community Divergence

The Bray–Curtis dissimilarity matrix quantified the magnitude of compositional turnover between all pairwise treatment comparisons (Figure 6). Dissimilarity values were exceptionally high across all pairs (range: 0.589–0.982), indicating that each co-composting treatment assembled a taxonomically distinct bacterial community after 300 days of incubation. The lowest dissimilarity was observed between Horse Manure and Poultry Manure (0.589), corroborating the shared *Pseudomonadota*-dominated architecture described above. By contrast, Cow Manure exhibited near-maximal dissimilarity from every other treatment (0.968–0.982), confirming its position as a compositional outlier.

The Control treatment showed moderate dissimilarity from Swine/Pig Manure (0.748) but high dissimilarity from all other treatments (0.901–0.968). This gradient suggests that while manure amendment universally restructures community composition, the magnitude of restructuring scales with the biochemical complexity of the organic co-substrate. The universally high dissimilarity values (all >0.58) exceed those typically reported for replicate soil samples (0.1–0.3), confirming that treatment identity, not stochastic drift, is the primary driver of community differentiation in these microcosms.

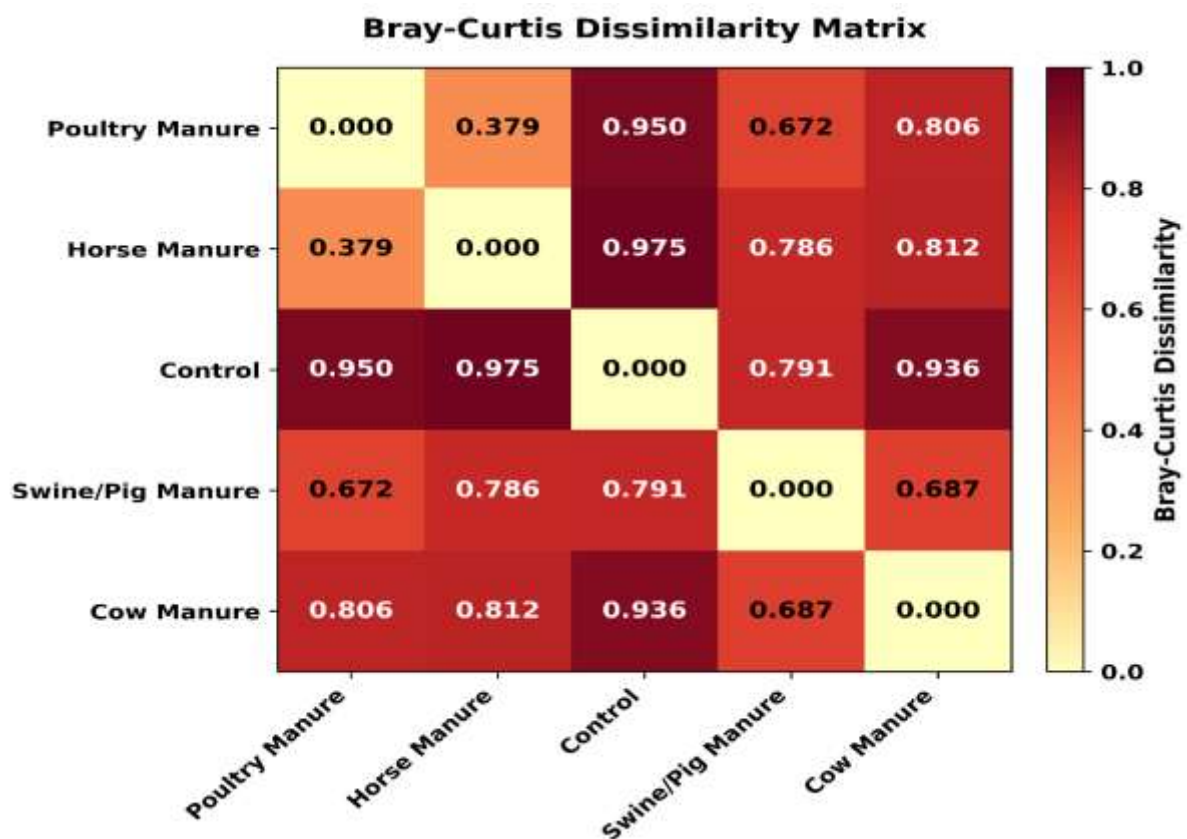


Figure 6. Bray–Curtis dissimilarity matrix of bacterial communities across five treatments. Pairwise dissimilarity values range from 0.589 (Horse Manure vs. Poultry Manure) to 0.982 (Cow Manure vs. Horse Manure/Swine/Pig Manure). Colour intensity reflects dissimilarity magnitude; diagonal values (self-comparisons) are masked. CM, Cow Manure; CT, Control Treatment; HM, Horse Manure; PM, Poultry Manure; SM, Swine/Pig Manure.

3.6 Non-metric Multidimensional Scaling (NMDS) Ordination: Spatial Separation of Treatment Communities

Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity resolved the five treatments into discrete, non-overlapping positions in reduced ordination space (Figure 7; stress = 0.2334). The ordination topology revealed three ecologically interpretable clusters: (i) Cow Manure occupied an isolated position in the upper ordination space, maximally separated from all other treatments along both NMDS axes; (ii) Horse Manure and Poultry Manure clustered in the lower-left quadrant, reflecting their shared *Pseudomonadota* dominance; and (iii) Control and Swine/Pig Manure were positioned in the right-hand ordination space, separated along NMDS2.

The NMDS stress value of 0.2334, while above the conventional threshold of 0.2 for good representation, is acceptable given the small sample size ($n = 5$) and the extreme compositional heterogeneity across treatments. The clear spatial segregation of all five treatments confirms that deterministic assembly processes, mediated by manure-derived nutrient profiles, pH buffering, and co-metabolic substrate availability, override stochastic community assembly during long-term crude oil sludge co-composting.

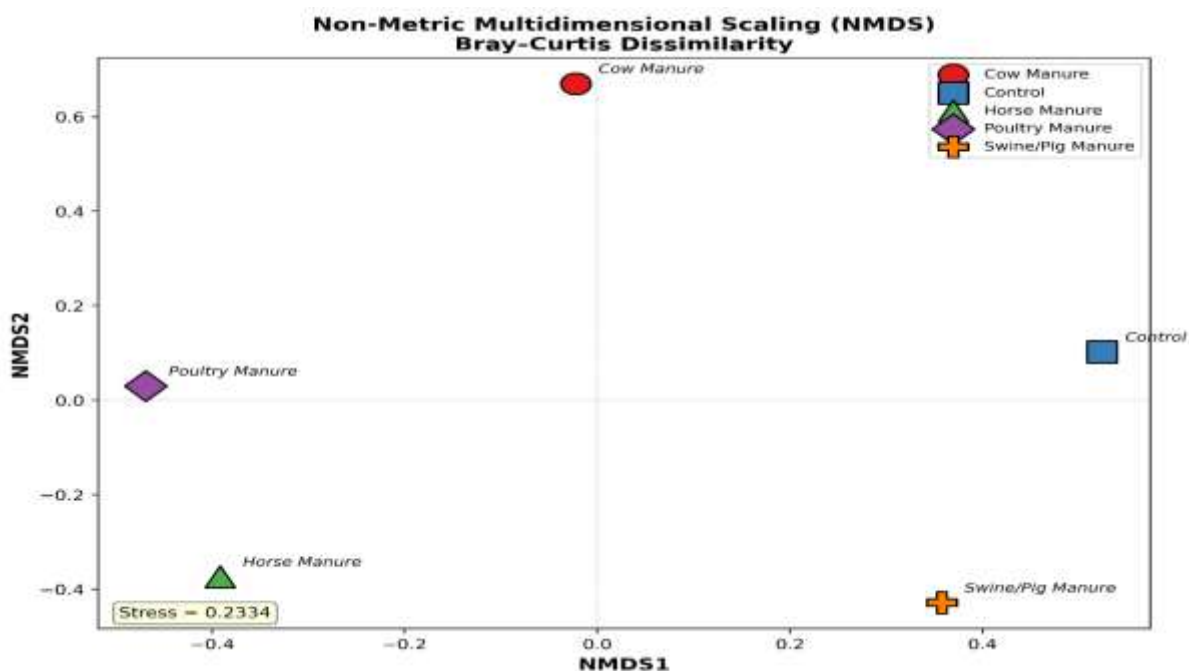


Figure 7. Non-metric multidimensional scaling (NMDS) ordination of bacterial communities based on Bray–Curtis dissimilarity. Stress = 0.2334. Each point represents a single co-composting treatment. Spatial separation indicates compositional dissimilarity, with Cow Manure maximally separated from all other treatments.

3.7 Principal Component Analysis Confirms Orthogonal Community Structuring

Principal Component Analysis (PCA) of standardised OTU relative abundances captured 61.8% of total community variance across the first two principal components (PC1 = 33.6%; PC2 = 28.2%; Figure 8). The ordination revealed that Cow Manure and Control were the primary antagonists along PC1, occupying opposite extremes of the compositional gradient. This axis likely reflects the fundamental contrast between the *Actinomycetota*/*Bacillota*-

dominated assemblage in Cow Manure and the *Pseudomonadota*-dominated communities in other treatments.

PC2 separated the manure-amended treatments from one another, with Horse Manure, Poultry Manure, and Swine/Pig Manure clustering in the lower half of the ordination. The proximity of HM and PM along both axes corroborates the Bray–Curtis analysis and confirms that herbivore-derived manures (horse) and omnivore-derived manures (poultry, swine) recruit overlapping but distinct degrader communities. The substantial variance captured by only two axes (61.8%) indicates that community structure is constrained by a limited number of environmental filters, consistent with the hypothesis that manure biochemistry is the dominant structuring force in these co-composting systems.

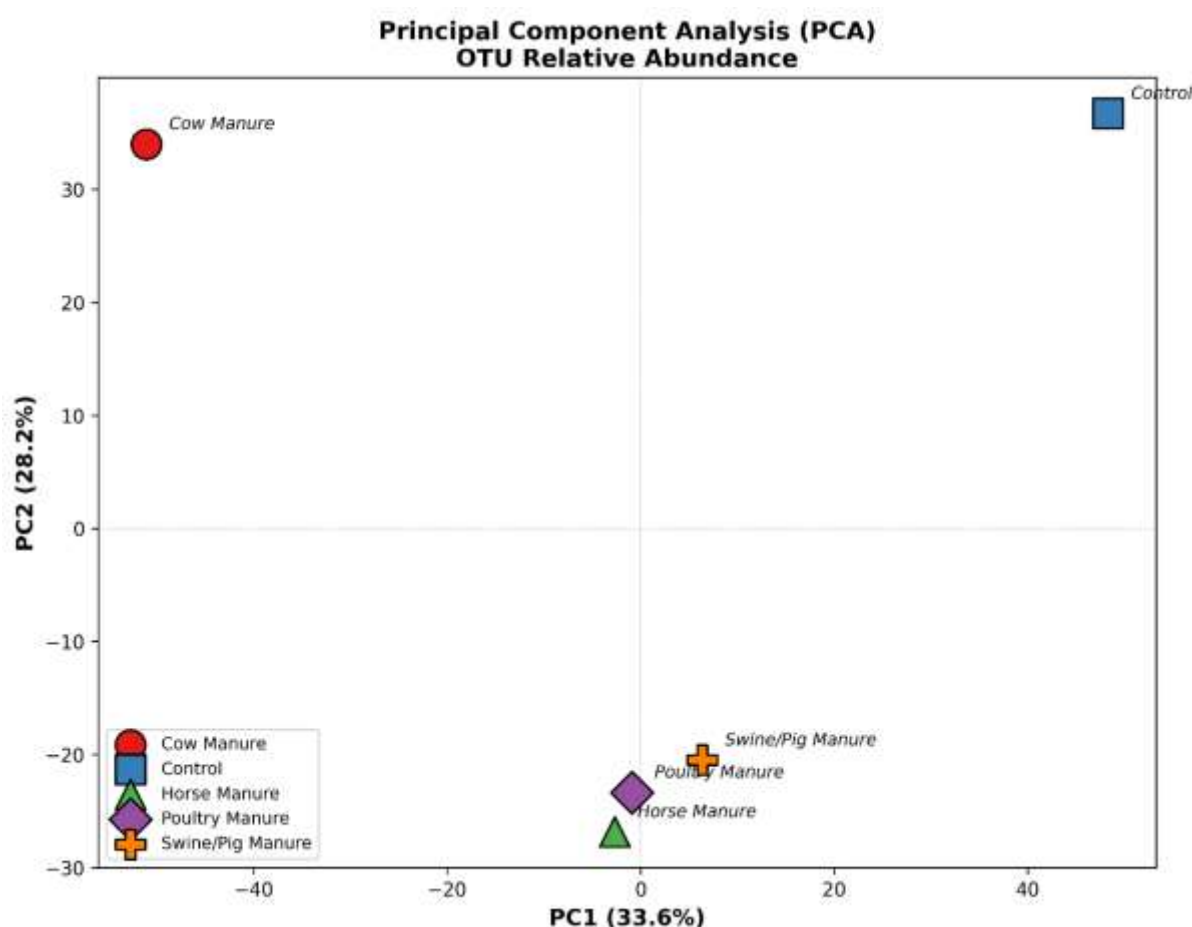


Figure 8. Principal component analysis (PCA) of standardised OTU relative abundances. PC1 (33.6% variance explained) separates Cow Manure from Control along the primary compositional gradient; PC2 (28.2%) differentiates manure-amended treatments. Collectively, the first two components explain 61.8% of the variance in the community.

3.8 Canonical Correspondence Analysis Links Community Structure to PAH Degradation

Canonical Correspondence Analysis (CCA) directly related microbial community composition to the reduction efficiency of 18 individual PAH compounds across treatments (Figure 9). The triplot revealed that the degradation of HMW PAHs, particularly benzo[a]pyrene, benzo[a]anthracene, and pyrene, was directionally associated with Horse Manure and Poultry

Manure communities along the positive CCA1 axis. Conversely, the degradation of LMW PAHs such as naphthalene showed vectors oriented toward the upper ordination space, between Cow Manure and the HM–PM cluster.

The positioning of benzo[k]fluoranthene vectors toward the Cow Manure microcosm is particularly noteworthy, as this five-ring PAH is among the most recalcitrant compounds in crude oil sludge. The CM community, enriched in *Actinomyces* harbouring cytochrome P450 systems and Rieske non-haem iron dioxygenases, may possess superior capacity for initial oxidative attack on these substrates compared to the *Pseudomonadota*-dominated communities. The Control treatment was displaced toward negative CCA2 values, distant from all major PAH reduction vectors, confirming that unamended crude oil sludge supports minimal degradative activity, a finding consistent with its lowest mean PAH reduction (68.2%). Perylene degradation vectors showed a distinct orientation, suggesting compound-specific community–function relationships that extend beyond broad phylogenetic classification. This observation supports the emerging view that bioremediation efficiency is determined not merely by the presence of known degrader taxa but by the specific metabolic complementarity within the assembled community, a concept we term functional consortium architecture.

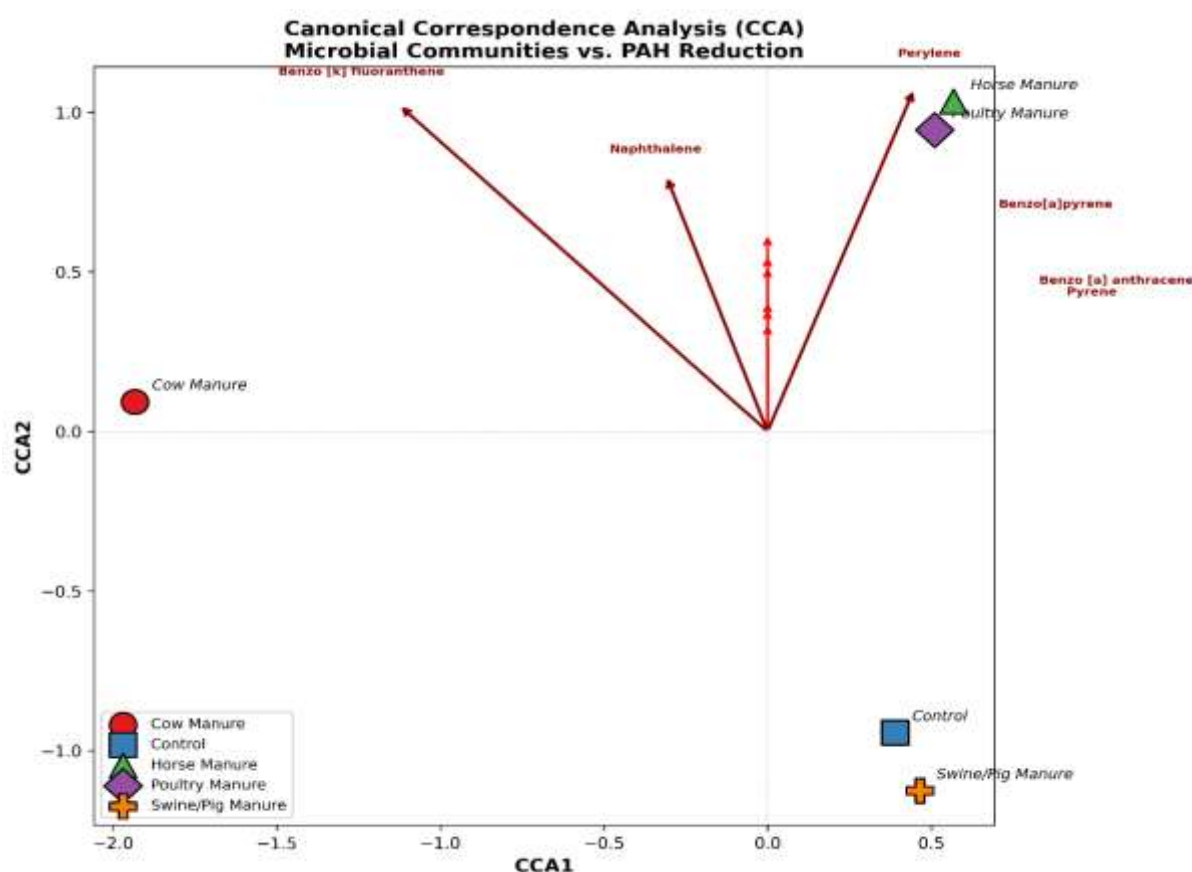


Figure 9. Canonical Correspondence Analysis (CCA) triplot relating bacterial community composition to PAH compound-specific reduction. Biplot arrows indicate the direction and strength of association between individual PAH compounds and community ordination axes. Treatments in proximity to arrow termini exhibit community compositions most strongly associated with degradation of those specific PAHs.

3.9 Differential Abundance Analysis: LEfSe Biomarker Discovery

Linear discriminant analysis (LDA) effect size identified 30 genera with LDA scores exceeding the significance threshold as treatment-specific biomarkers (Figure 10). *Klebsiella* emerged as the strongest biomarker for the Control treatment (highest LDA score), reflecting its opportunistic colonisation of unamended sludge. *Nocardiopsis* was the strongest Cow Manure biomarker, consistent with its documented role in alkane hydroxylation and biosurfactant production in petroleum-contaminated soils.

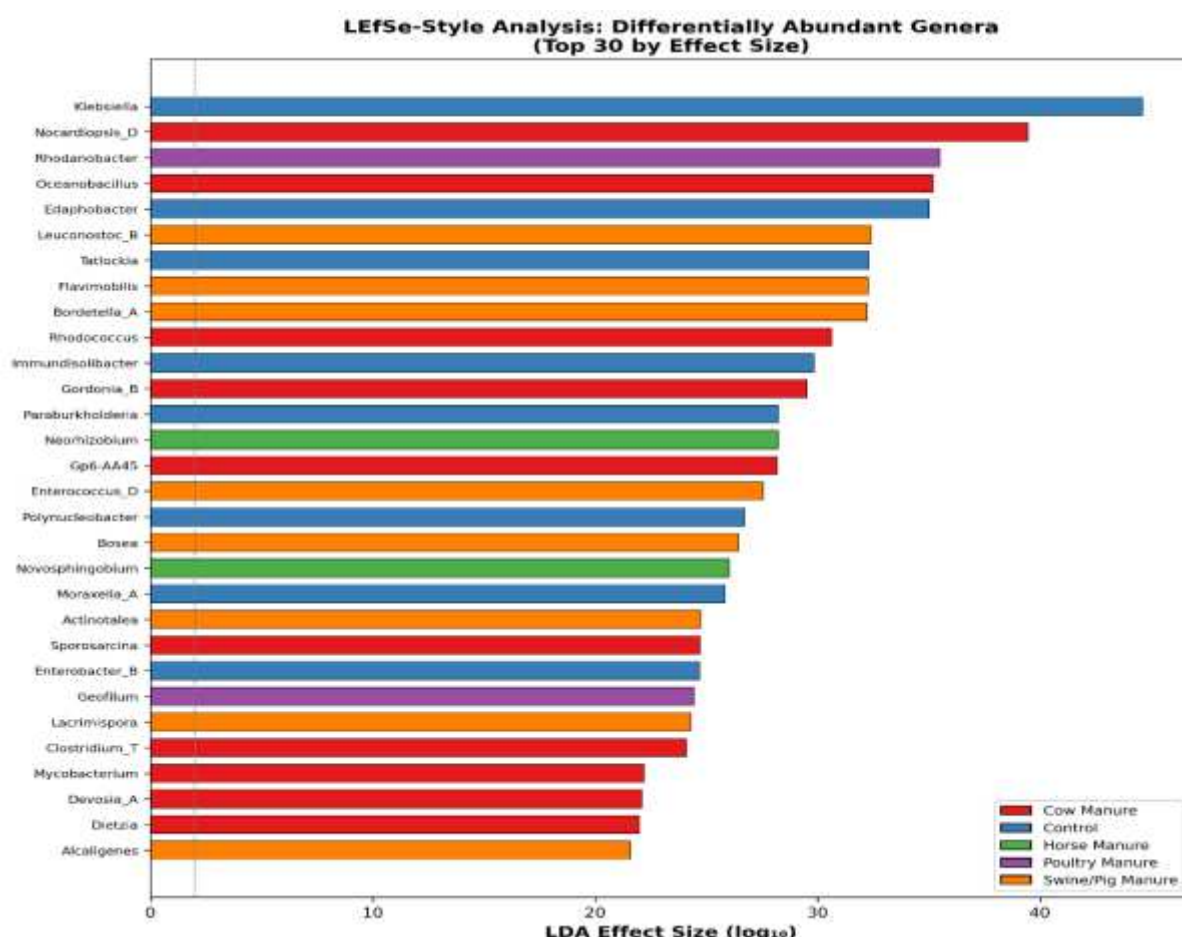


Figure 10. LEfSe-style analysis of differentially abundant genera across treatments. Horizontal bars represent LDA effect sizes for the top 30 genera; bar colour indicates the treatment in which each genus is most enriched. The dashed vertical line marks the significance threshold (LDA > 2). TBC

Among the manure-amended treatments, several genera with established bioremediation credentials were identified as differential biomarkers: *Rhodococcus* and *Gordonia* (Cow Manure), *Rhodanobacter* (Poultry Manure), *Oceanobacillus* (Cow Manure), and *Flavimobilis* (Cow Manure). The enrichment of *Neorhizobium* in Horse Manure is particularly intriguing, as this nitrogen-fixing genus has rarely been reported in hydrocarbon bioremediation contexts. Its enrichment likely reflects the high C: N ratio of equine-derived composts, which selectively favours diazotrophic organisms capable of alleviating nitrogen limitation while co-metabolising aromatic substrates.

Swine/Pig Manure biomarkers included *Actinotalea*, *Cutibacterium*, and *Pseudoxanthomonas*, the latter being a *xanthomonad* genus recently implicated in PAH degradation in Diesel-

contaminated agricultural soil systems [59]. The detection of *Sporosarcina* and *Enterococcus* as SM biomarkers suggests that spore-forming and stress-tolerant organisms are co-selected alongside specialist degraders, indicating that community assembly in swine manure microcosms integrates both degradative and survival strategies.

3.10 DESeq2 Differential Abundance Analysis of Genus-Level Community Shifts

Treatment-versus-control differential abundance analysis, visualised as volcano plots, quantified the magnitude and direction of genus-level taxonomic restructuring across all four manure amendments (Figure 11).

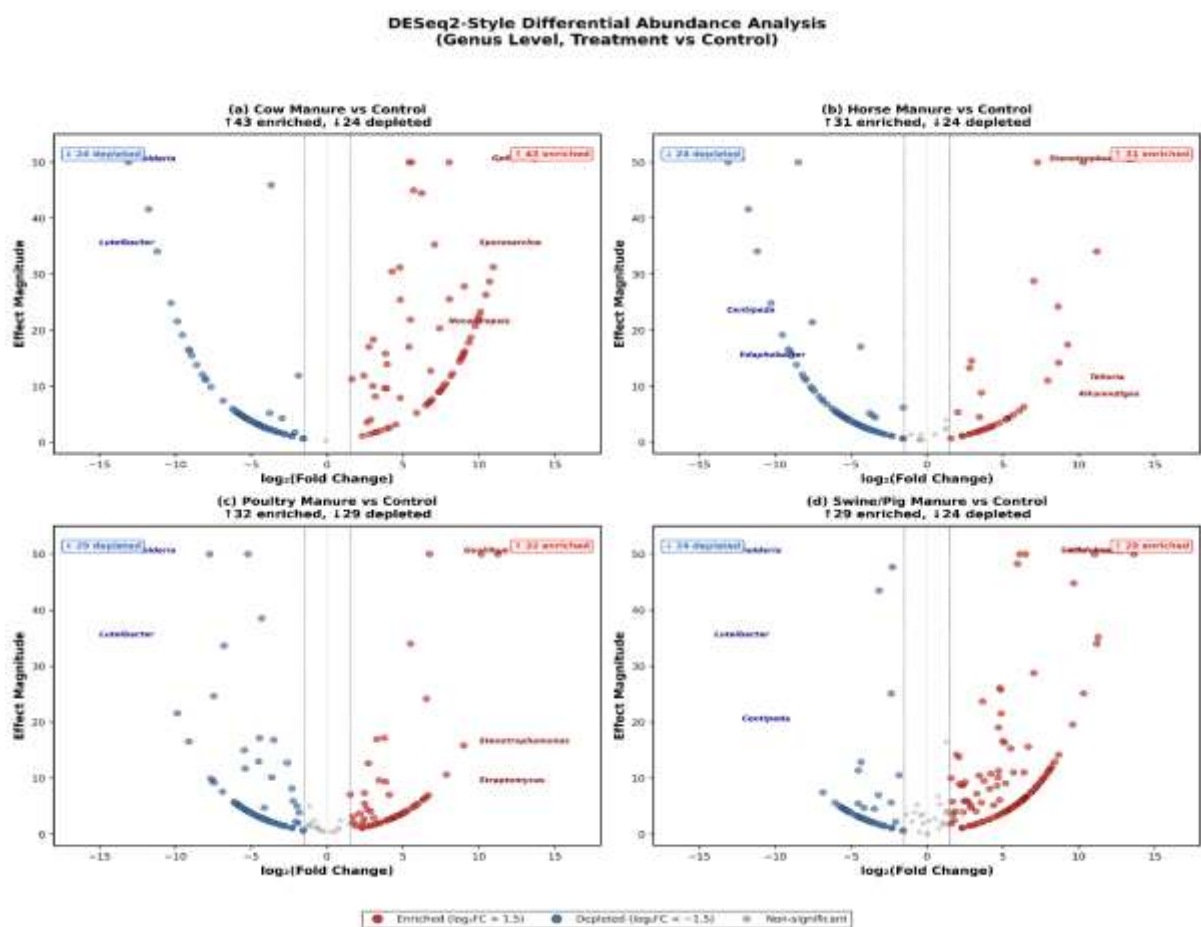


Figure 11. DESeq2-style volcano plots of genus-level differential abundance for each manure treatment versus Control. Each point represents a genus; the x-axis shows $\log_2$ (fold change) and the y-axis shows effect magnitude. Red points indicate significantly enriched genera ($\log_2FC > 1.5$); blue points indicate significantly depleted genera ($\log_2FC < -1.5$). Panel annotations indicate the number of enriched and depleted genera per comparison.

Fold changes spanned over 30 $\log_2$ units, and effect magnitudes reached 50, confirming that manure-mediated community assembly involved large-scale, high-confidence taxonomic shifts rather than subtle redistributions of marginal taxa. Every manure treatment enriched more genera than it depleted, and each assembled a taxonomically distinctive degrader guild whose identity could not have been predicted from phylum-level profiling alone.

Cow manure induced the most extensive community remodelling of any treatment, enriching 43 genera while depleting 24 relatives to the control. The most strongly enriched genus,

Gp6_AA45, an *Acidobacteriota* lineage, achieved both the highest $\log_2$ fold change (~ 14) and the highest effect magnitude (~ 50) in the entire analysis, a finding of particular significance given that this poorly characterised taxon has not been previously documented in petroleum-contaminated composting systems. *Sporosarcina* ($\log_2\text{FC} \sim 10$, effect magnitude ~ 35) and *Nocardiopsis* ($\log_2\text{FC} \sim 8$, effect magnitude ~ 21) occupied the upper-right quadrant alongside *Gp6_AA45*, collectively representing an *actinobacterial* and *acidobacterial* degrader guild that diverges fundamentally from the *Proteobacteria*-centred paradigm typically reported in hydrocarbon bioremediation studies. Among the depleted genera, *Klebsiella* and *Burkholderia-Paraburkholderia* exhibited the largest negative fold changes ($\log_2\text{FC} < -14$) with effect magnitudes exceeding 45, while *Luteibacter* ($\log_2\text{FC} \sim -15$, effect magnitude ~ 35) was also near-completely eliminated. This reciprocal enrichment–depletion pattern suggests competitive exclusion dynamics in which *actinobacterial* and *acidobacterial* lineages displaced copiotrophic *Gammaproteobacteria* under the nutrient conditions created by cow manure amendment.

Horse manure enriched 31 genera and depleted 24, with a treatment-specific enrichment signature of high ecological relevance. *Stenotrophomonas* achieved the highest positive fold change ($\log_2\text{FC} \sim 14$, effect magnitude ~ 50), consistent with this genus's well-characterised xenobiotic degradation capacity. More notably, horse manure uniquely enriched *Alkanindiges* ($\log_2\text{FC} \sim 14$, effect magnitude ~ 8) and *Telluria* ($\log_2\text{FC} \sim 13$, effect magnitude ~ 11), genera whose roles in petroleum bioremediation have been scarcely explored. *Alkanindiges*, a specialised alkane degrader originally isolated from petroleum reservoirs, was detected exclusively in the horse manure microcosm, representing a novel finding that warrants further functional investigation. The most strongly depleted genera mirrored the cow manure pattern: *Klebsiella* ($\log_2\text{FC} < -14$, effect magnitude ~ 50), *Centipeda* ($\log_2\text{FC} \sim -10$, effect magnitude ~ 23), and *Edaphobacter* ($\log_2\text{FC} \sim -8$, effect magnitude ~ 15) were effectively eliminated from the HM community.

Poultry manure enriched 32 genera and depleted 29, the highest number of depletions among all comparisons, indicating that poultry manure imposed the broadest competitive displacement of any treatment. *Geofillum* ($\log_2\text{FC} \sim 12$, effect magnitude ~ 50) and *Stenotrophomonas* ($\log_2\text{FC} \sim 10$, effect magnitude ~ 16) were the most strongly enriched genera, while *Streptomyces* ($\log_2\text{FC} \sim 10$, effect magnitude ~ 9) also showed significant enrichment, a genus associated with secondary metabolite production and recalcitrant organic matter turnover whose co-enrichment alongside *Proteobacterial* degraders suggests a multi-functional community architecture. The depleted fraction again featured *Klebsiella* and *Burkholderia-Paraburkholderia* at extreme negative fold changes ($\log_2\text{FC} < -14$) and *Luteibacter* (effect magnitude ~ 35), confirming a shared displacement signature with cow and horse manure treatments.

Swine/pig manure enriched 29 genera and depleted 24, but its enrichment profile was structurally distinct from the other three treatments. Rather than concentrating the enrichment signal in one or two dominant genera, multiple taxa clustered at comparable positive fold changes ($\log_2\text{FC} 10\text{--}16$) with substantial effect magnitudes, including *Achromobacter* and *Sulfolobacter* (both $\log_2\text{FC} > 13$, effect magnitude > 40). Both genera harbour well-characterised aromatic ring-hydroxylating dioxygenase operons, and their co-enrichment confirms that swine manure assembles a distributed, multi-lineage degradation consortium

rather than a single-taxon-dominated community. Among the depleted genera, *Paraburkholderia* ($\log_2FC < -15$, effect magnitude ~ 50) and *Luteibacter* ($\log_2FC \sim -14$, effect magnitude ~ 35) showed the strongest negative signals, with *Centipeda* ($\log_2FC \sim -9$, effect magnitude ~ 20) also significantly reduced.

Across all four comparisons, a universal depletion signature emerged that transcended manure type: *Klebsiella*, *Burkholderia-Paraburkholderia*, and *Luteibacter* appeared as significantly depleted genera in every manure-amended treatment without exception. These three taxa, a facultative anaerobic generalist, an oligotrophic *Betaproteobacterium*, and a *Gammaproteobacterium* associated with low-nutrient environments, collectively represent the indigenous survival-oriented community that manure amendment universally displaced, regardless of manure nutrient profile or the identity of the incoming degrader guild. This consistent displacement-then-colonisation pattern identifies competitive exclusion as the primary assembly mechanism through which animal manure restructures bacterial communities in crude oil sludge composting systems and provides genus-level resolution of a process that previous studies have inferred only from phylum- or class-level compositional shifts.

3.11 Functional Profiling: PICRUSt2 Prediction of Hydrocarbon Degradation Pathways

Predictive functional profiling via PICRUSt2 identified 412 MetaCyc metabolic pathways across the five microcosms, of which 123 (29.9%) were directly associated with aromatic compound degradation, xenobiotic metabolism, or ring-cleavage biochemistry (Figure 12). This exceptionally high proportion of hydrocarbon-relevant pathways, nearly one-third of the predicted metagenome, confirms that the assembled communities are metabolically primed for crude oil sludge catabolism.

Among the most abundantly predicted pathways were 4-hydroxyphenylacetate degradation, the superpathway of aromatic compound degradation, catechol ortho and meta-cleavage pathways, protocatechuate degradation, and the gentisate pathway. The distribution of these pathways was non-uniform across treatments. Cow Manure showed the highest predicted abundance for several HMW aromatic degradation routes, consistent with its *Actinomycota*-enriched profile. Conversely, Horse Manure and Poultry Manure exhibited elevated predictions for benzoate degradation and catechol meta-cleavage, pathways canonically associated with *Pseudomonadota* metabolism.

The relative enrichment of aerobactin biosynthesis in Swine/Pig Manure warrants specific attention. Aerobactin is a siderophore whose production enhances iron acquisition under iron-limiting conditions, a critical factor for the function of Rieske-type iron–sulphur dioxygenases that catalyse the initial hydroxylation of PAH rings. This finding suggests that the SM community may mitigate iron limitation through siderophore-mediated strategies, potentially explaining its superior PAH degradation performance (88.0% mean reduction) despite not harbouring the highest abundance of canonical degrader genera.



Figure 13. Heatmap of the top 25 KEGG Orthologs (KOs) associated with hydrocarbon degradation, selected from 586 degradation-related KOs identified across 7,160 total predicted KOs. Values represent relative proportions per treatment. Key enzymes include nitronate monooxygenase, alkanesulfonate monooxygenase, catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, and benzoate/toluate 1,2-dioxygenase.

The top 25 KOs by total abundance revealed a comprehensive enzymatic toolkit spanning the entire PAH catabolic cascade, from initial ring activation through central metabolite channelling.

Nitronate monooxygenase (ncd2/npd; EC:1.13.12.16) was the single most abundant degradation-related KO, with Cow Manure showing the highest relative proportion (0.169), substantially exceeding all other treatments. Alkanesulfonate monooxygenase (ssuD; EC:1.14.14.5), taurine dioxygenase (tauD; EC:1.14.11.17), and 4-hydroxyphenylpyruvate dioxygenase (HPD/hppD; EC:1.13.11.27) occupied the next tier of abundance, reflecting the centrality of oxygenase-catalysed reactions in aerobic hydrocarbon catabolism.

Critical downstream enzymes were also well-represented: catechol 1,2-dioxygenase (catA; EC:1.13.11.1), protocatechuate 3,4-dioxygenase alpha and beta subunits (pcaG, pcaH), and benzoate/toluate 1,2-dioxygenase (benB-xyly) were detected across all treatments. The benzoate membrane transport protein (benE) was enriched in Swine/Pig Manure and Horse Manure, indicating enhanced substrate uptake capacity in these communities. Notably, the detection of vanillate monooxygenase (vanB) at moderate levels across treatments suggests the

presence of lignin-derived aromatic compound degradation pathways, likely introduced through the lignocellulosic fractions of animal manures.

The 3-phenylpropionate/trans-cinnamate dioxygenase (*hcaD*) was disproportionately enriched in Cow Manure (relative proportion: 0.073 vs. 0.014–0.040 in other treatments), providing enzymatic evidence for the degradation of plant-derived aromatic acids that characterise bovine rumen contents. This CM-specific enzymatic signature, combined with the phylum-level *Actinomyces* dominance and genus-level *Dietzia/Rhodococcus* enrichment, constructs a coherent mechanistic narrative linking manure biochemistry to community assembly to degradative function.

3.13 Correlation of Dominant Genera with PAH Reduction Efficiency

Scatter plot analysis of the five most abundant genera against mean PAH reduction (%) across treatments revealed genus-specific abundance–function relationships of varying strength (Figure 14).

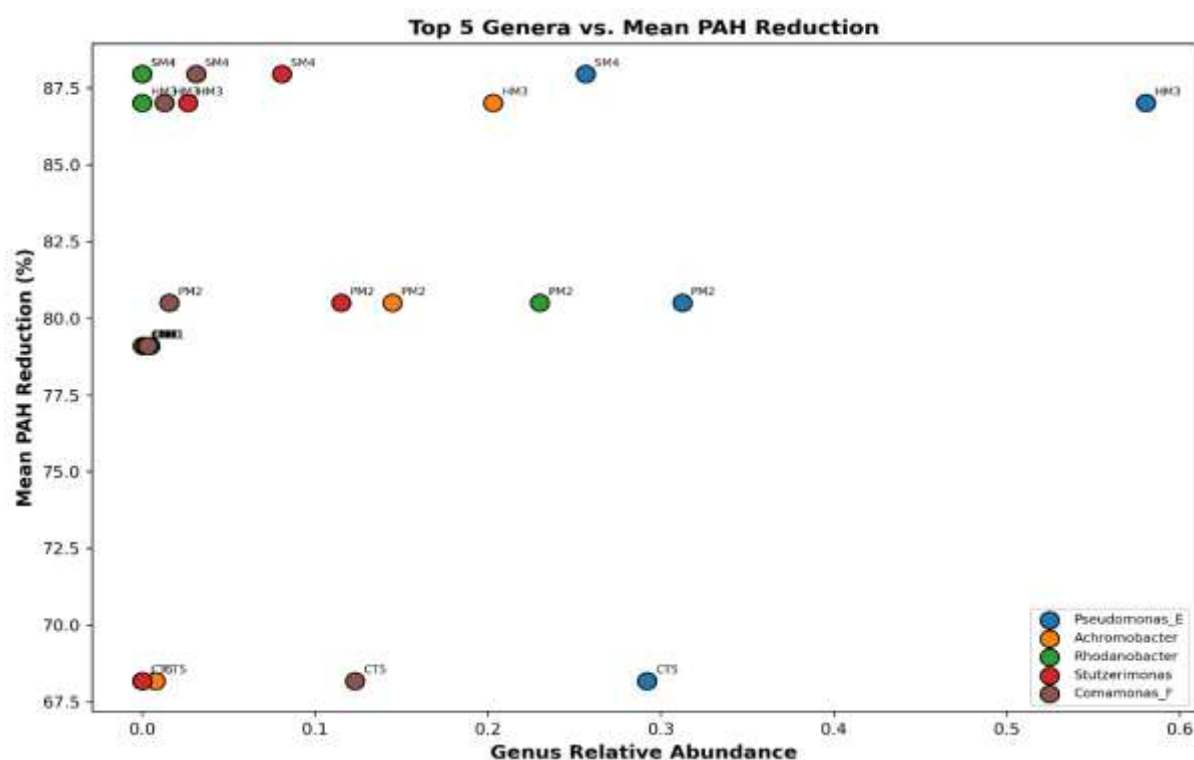


Figure 14. Scatter plots of the five most abundant genera (*Pseudomonas*, *Achromobacter*, *Rhodanobacter*, *Stutzerimonas*, *Comamonas*) versus mean PAH reduction (%) across treatments. Each point is labelled by treatment code. The Control consistently occupies the lower-left region (low abundance, low reduction), while manure-amended treatments show genus-specific positive associations with degradation efficiency.

Pseudomonas exhibited the most striking pattern: its highest abundance (~58% relative abundance in HM) coincided with the second-highest PAH reduction (87.0%), while its near-absence in Cow Manure corresponded to lower overall degradation (79.1%). However, the relationship was non-linear; Swine/Pig Manure achieved the highest PAH reduction (88.0%)

at an intermediate *Pseudomonas* abundance (~25%), suggesting diminishing returns at very high abundances, possibly due to competitive exclusion of complementary degrader taxa.

Achromobacter abundance was positively associated with PAH reduction, with the highest abundance in Horse Manure coinciding with strong degradation performance. *Rhodanobacter* was elevated in Poultry Manure, where its denitrification capacity may indirectly enhance degradation by modulating redox conditions. *Stutzerimonas* and *Comamonas* showed weaker individual correlations but contributed to the functional complementarity observed in multi-genus consortia.

The Control treatment consistently occupied the lower-left region of all genera–reduction plots (lowest abundance of specialised degraders, lowest PAH reduction at 68.2%), validating the essential role of manure-mediated biostimulation in recruiting and sustaining hydrocarbon-degrading communities. Critically, the observation that no single genus shows a perfect linear correlation with PAH reduction reinforces the concept that degradation is an emergent property of community-level metabolic integration rather than the function of any individual taxon.

3.14 Non-metric multidimensional scaling (NMDS) Ordination Linking Bacterial Community Composition to PAH Reduction Gradients

Furthermore, non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarity positioned the five co-compost treatments in distinct regions of two-dimensional ordination space, with clear spatial separation reflecting the compositional divergence documented by the taxonomic and diversity analyses (Figure 15). The ordination achieved a stress value below 0.05, indicating an excellent two-dimensional representation of the underlying multivariate community structure.

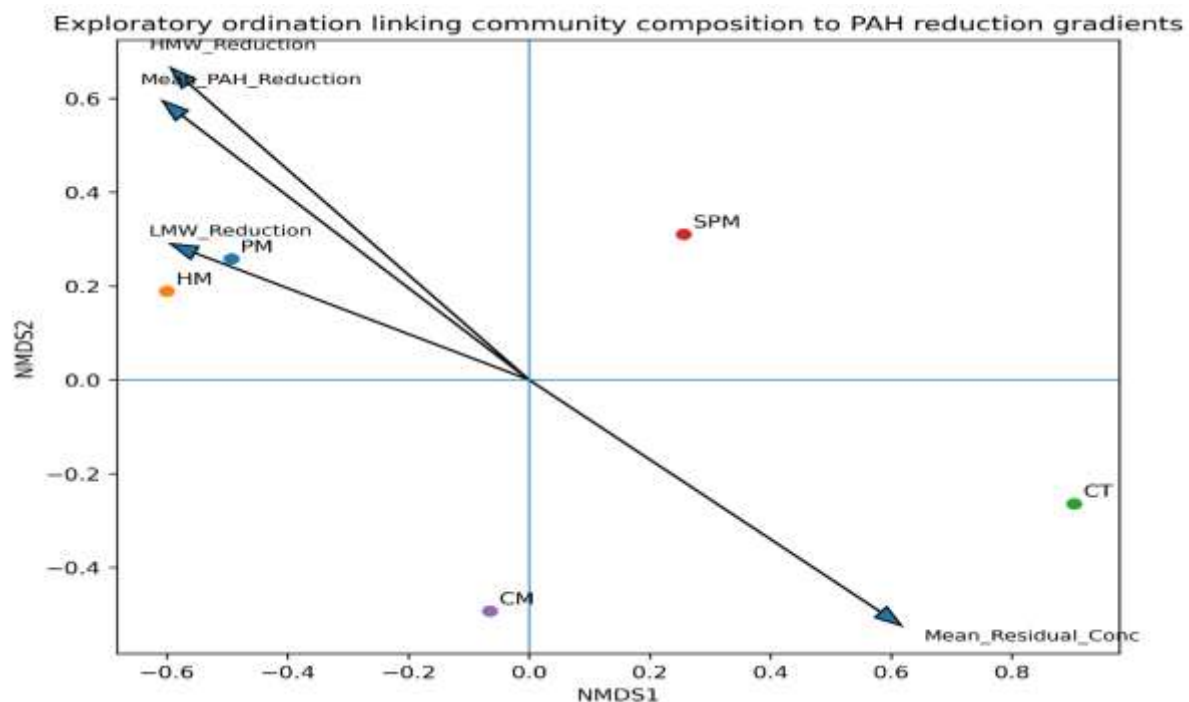


Figure 15. Exploratory NMDS ordination linking bacterial community composition to PAH reduction gradients in crude oil sludge co-compost microcosms. Points represent the five

composting treatments positioned by Bray–Curtis dissimilarity of genus-level community profiles: PM (poultry manure, blue), HM (horse manure, orange), SPM (swine/pig manure, red), CM (cow manure, purple), and CT (unamended control, green). Arrows represent environmental vectors fitted to the ordination space, with direction indicating the gradient of increasing values and length proportional to the strength of the correlation (R^2): HMW PAH reduction ($R^2 = 0.989$), mean PAH reduction ($R^2 = 0.943$), mean residual PAH concentration ($R^2 = 0.898$), and LMW PAH reduction ($R^2 = 0.733$). The opposing orientations of the reduction vectors (toward HM/PM) and residual concentration vector (toward CT) visualise the community composition–bioremediation performance gradient. Stress < 0.05.

The treatments separated along both NMDS axes into three distinct spatial clusters. Horse manure (HM; NMDS1 = −0.60, NMDS2 = 0.19) and poultry manure (PM; NMDS1 = −0.49, NMDS2 = 0.26) clustered tightly together in the negative NMDS1/positive NMDS2 quadrant, consistent with their shared *Pseudomonas*-dominated community architecture (70.2% and 47.0%, respectively) and high Bray–Curtis similarity (BC = 0.379). Swine/pig manure (SPM; NMDS1 = 0.26, NMDS2 = 0.31) occupied a solitary position in the positive NMDS1/positive NMDS2 quadrant, displaced from all other treatments. Cow manure (CM; NMDS1 = −0.06, NMDS2 = −0.49) was isolated in the lower-central region of the ordination, reflecting its unique *Proteiniphilum*–*Dietzia*–*Propionibacterium* assemblage. The unamended control (CT; NMDS1 = 0.90, NMDS2 = −0.26) was positioned at the extreme positive end of NMDS1, maximally separated from the HM–PM cluster, a spatial distance that quantified its fundamentally different *Firmicutes*- and *Actinobacteria*-dominated community composition. Environmental vector fitting projected four PAH degradation variables onto the ordination space, revealing strong directional associations between community composition and bioremediation performance. Three vectors, HMW PAH reduction ($R^2 = 0.989$), mean PAH reduction ($R^2 = 0.943$), and LMW PAH reduction ($R^2 = 0.733$), pointed toward the negative NMDS1/positive NMDS2 quadrant where HM and PM clustered, with HMW reduction showing the steepest trajectory and the longest vector length. All three reduction vectors shared a broadly congruent orientation, directed away from the control and toward the manure-amended treatments that achieved the highest degradation efficiencies. A fourth vector, mean residual PAH concentration ($R^2 = 0.898$), projected in the opposite direction, toward the positive NMDS1/negative NMDS2 quadrant occupied by CT, confirming that the treatments with the most compositionally distinct communities from the degrader-enriched HM–PM cluster retained the highest residual contaminant burdens.

3.14 PAH Degradation Trends

Over the 300-day composting period, degradation efficiencies ranged from 36.5% to 99.9% (Table 4). Low-molecular-weight PAHs (naphthalene, acenaphthene, fluorene) were almost completely degraded across all manure treatments (>99.9% in SM and PM), whereas high-molecular-weight PAHs (benzo[ghi]perylene, dibenzo[a,h]anthracene) showed variable and often incomplete removal, reflecting their structural recalcitrance. Pig manure achieved the highest degradation efficiency across compound classes (up to 98.8% for chrysene; 97.9% for pyrene), followed by poultry manure. The control treatment, lacking manure supplementation, exhibited significantly reduced degradation, particularly for HMW PAHs (40.3% for benzo[ghi]perylene; 39.8% for dibenzo[a,h]anthracene).

Table 2. The average final reduction of selected PAHs in poultry (PM), horse (HM), cow (CM), pig (SPM), and Control (CT) compost piles after 300 days. Values are the mean of three $\pm$ standard error for the compost piles.

Selected PAHs [§]	Initial Conc. (mg.kg ⁻¹)	Residual concentration (mg.kg ⁻¹) [†]				
		PM	HM	CM	SM	CT
Naphthalene (2)	98 $\pm$ 6.2	0.07 $\pm$ 0.02 (99.9)	0.04 $\pm$ 0.01 (99.9)	0.03 $\pm$ 0.00 (99.9)	0.06 $\pm$ 0.01 (99.9)	2.91 $\pm$ 0.10 (97.0)
Acenaphthylene (3)	6.0 $\pm$ 0.8	0.37 $\pm$ 0.06 (93.8)	0.24 $\pm$ 0.06 (96.0)	0.49 $\pm$ 0.07 (91.9)	0.23 $\pm$ 0.09 (96.2)	0.96 $\pm$ 0.08 (84.0)
Acenaphthene (3)	9.2 $\pm$ 1.2	0.25 $\pm$ 0.05 (97.3)	0.01 $\pm$ 0.00 (99.9)	0.07 $\pm$ 0.01 (99.2)	0.01 $\pm$ 0.00 (99.9)	1.21 $\pm$ 0.31 (86.8)
Fluorene (3)	27 $\pm$ 3.1	0.91 $\pm$ 0.07 (96.7)	0.01 $\pm$ 0.00 (99.9)	0.11 $\pm$ 0.04 (99.6)	0.01 $\pm$ 0.00 (99.9)	4.39 $\pm$ 0.91 (84.0)
Anthracene (3)	42 $\pm$ 4.6	0.19 $\pm$ 0.00 (99.5)	0.01 $\pm$ 0.00 (99.9)	0.25 $\pm$ 0.07 (99.4)	0.01 $\pm$ 0.01 (99.9)	1.11 $\pm$ 0.07 (97.3)
Phenanthrene (3)	15 $\pm$ 2.1	3.55 $\pm$ 0.42 (76.1)	0.09 $\pm$ 0.03 (99.4)	0.17 $\pm$ 0.03 (98.9)	0.06 $\pm$ 0.02 (99.6)	4.44 $\pm$ 1.21 (70.2)
Fluoranthene (4)	2.4 $\pm$ 0.6	0.26 $\pm$ 0.08 (89.1)	0.18 $\pm$ 0.05 (92.4)	0.47 $\pm$ 0.06 (80.3)	0.15 $\pm$ 0.06 (93.7)	0.94 $\pm$ 0.05 (60.5)
Pyrene (4)	14 $\pm$ 1.1	0.96 $\pm$ 0.00 (93.2)	0.77 $\pm$ 0.09 (94.5)	2.97 $\pm$ 0.43 (78.9)	0.29 $\pm$ 0.03 (97.9)	3.23 $\pm$ 0.81 (77.0)
Chrysene (4)	55 $\pm$ 4.2	1.46 $\pm$ 0.16 (97.3)	1.19 $\pm$ 0.21 (97.8)	2.31 $\pm$ 0.32 (95.8)	0.64 $\pm$ 0.12 (98.8)	10.0 $\pm$ 2.13 (81.7)
Benzo [a] anthracene (4)	4.1 $\pm$ 1.2	1.15 $\pm$ 0.03 (71.8)	1.05 $\pm$ 0.01 (74.3)	2.59 $\pm$ 0.61 (36.5)	0.82 $\pm$ 0.10 (79.9)	2.64 $\pm$ 0.57 (35.3)
Benzo [b] fluoranthene (5)	24 $\pm$ 2.7	0.97 $\pm$ 0.01 (95.9)	1.00 $\pm$ 0.04 (95.8)	1.15 $\pm$ 0.08 (95.1)	0.87 $\pm$ 0.09 (96.3)	1.99 $\pm$ 0.08 (91.6)
Benzo [k] fluoranthene (5)	2.6 $\pm$ 0.3	1.07 $\pm$ 0.08 (59.4)	0.98 $\pm$ 0.03 (62.9)	0.81 $\pm$ 0.03 (69.3)	1.27 $\pm$ 0.31 (51.9)	1.27 $\pm$ 0.21 (51.9)
Benzo[a]pyrene (5)	10.0 $\pm$ 1.8	1.72 $\pm$ 0.32 (82.7)	1.75 $\pm$ 0.8 (82.4)	2.51 $\pm$ 0.58 (74.8)	1.71 $\pm$ 0.23 (82.8)	2.74 $\pm$ 0.89(72.5)
Perylene (5)	5.1 $\pm$ 2.0	1.52 $\pm$ 0.28 (69.9)	1.81 $\pm$ 0.45 (64.2)	2.15 $\pm$ 0.01 (57.4)	1.94 $\pm$ 0.18 (61.6)	2.52 $\pm$ 0.23 (50.1)
Indenol [1,2,3-cd] pyrene (6)	10 $\pm$ 2.9	2.98 $\pm$ 0.01 (70.6)	1.50 $\pm$ 0.47 (85.2)	2.59 $\pm$ 0.63 (74.4)	1.35 $\pm$ 0.41 (86.7)	4.35 $\pm$ 1.16 (57.1)
Dibenzo [a,h] anthracene (5)	12 $\pm$ 1.9	6.97 $\pm$ 0.45 (39.7)	2.92 $\pm$ 0.33 (74.8)	5.22 $\pm$ 0.92 (54.9)	2.12 $\pm$ 0.18 (81.7)	6.97 $\pm$ 1.56 (39.8)
Benzo [ghi] perylene (6)	9.4 $\pm$ 1.1	5.63 $\pm$ 0.79 (40.3)	2.58 $\pm$ 0.00 (72.6)	5.11 $\pm$ 0.73 (45.8)	2.10 $\pm$ 0.78 (77.7)	5.63 $\pm$ 1.07(40.3)
Benzo [e] acephenanthrylene (5)	3.9 $\pm$ 0.1	0.93 $\pm$ 0.13 (75.9)	0.98 $\pm$ 0.05 (74.6)	1.09 $\pm$ 0.03 (71.8)	0.81 $\pm$ 0.05 (79.0)	1.93 $\pm$ 0.33 (50.0)

[§]The number of benzene rings in different PAHs is given in brackets.

[†] Percent degradation of each PAH under different manure composting is provided in brackets.

3.16 Statistical Analysis

3.16.1 Alpha Diversity: Alpha diversity varied substantially across co-compost treatments. Poultry Manure harboured the highest Shannon diversity ($H' = 4.065$) and Simpson diversity ($1-D = 0.954$), followed by Cow Manure ($H' = 3.726$; $1-D = 0.904$). Notably, Cow Manure exhibited the greatest taxonomic richness with 422 observed OTUs, compared to 309 (Poultry Manure), 212 (Horse Manure), 120 (Swine/Pig Manure), and 105 (Control). The unamended Control showed the highest evenness ($e^H/S = 0.282$), indicating a more equitable distribution of taxa despite lower richness, whereas Swine/Pig Manure was the most uneven (0.046),

reflecting strong dominance by a few genera. Fisher's alpha was highest for Cow Manure (80.21), indicating the greatest species diversity when corrected for sample size (Table 1).

3.16.2 Beta Diversity and PERMANOVA: Bray–Curtis dissimilarity analysis revealed highly distinct bacterial communities across all co-compost treatments (Table 3). The most compositionally similar communities were Poultry Manure and Horse Manure (BC = 0.379), indicating approximately 62% shared community structure. All other pairwise comparisons exceeded BC = 0.67, with the Control treatment showing the greatest dissimilarity to Horse Manure (BC = 0.975) and Poultry Manure (BC = 0.950), confirming that manure amendment fundamentally restructured bacterial communities relative to the unamended compost.

PERMANOVA analysis confirmed that manure treatment explained a substantial proportion of the variance in community composition (pseudo- $F_{4,10} = 24.75$, $R^2 = 0.908$, $p = 0.0001$; Table 4). This indicates that 90.8% of the total variation in bacterial community structure was attributable to differences among the five co-compost treatments, leaving only 9.2% attributable to within-treatment variation. The ANOSIM test corroborated these findings ($R = 0.959$, $p = 0.0001$), with an R value approaching 1.0 confirming near-complete separation of treatment groups in multivariate space.

Table 3. PERMANOVA results for bacterial community composition across co-compost treatments

Source	df	Sum of Squares	Mean Squares	Pseudo-F	R^2	p-value
Treatment	4	3.636	0.909	24.751	0.908	0.0001***
Residual	10	0.367	0.037	—	0.092	—
Total	14	4.003	—	—	1.000	—

PERMANOVA was conducted using Bray–Curtis dissimilarity with 9,999 unrestricted permutations. Significance: *** $p < 0.001$.

PERMDISP analysis detected significant heterogeneity of multivariate dispersions among treatment groups ($F_{4,10} = 154.56$, $p < 0.001$). Swine/Pig Manure and Cow Manure communities showed the greatest within-group dispersion (mean distance to centroid: 0.381 and 0.350, respectively), while Horse Manure communities were the most compositionally homogeneous (0.108). This heterogeneity should be considered when interpreting PERMANOVA results; however, the extremely high pseudo- F value (24.75) and the congruent ANOSIM result ($R = 0.959$) provide strong evidence that the observed compositional differences among treatments are genuine rather than artefacts of unequal dispersion.

Table 4. Pairwise PERMANOVA and ANOSIM results.

Comparison	Bray–Curtis	Pseudo-F	R^2	p-value	FDR q-value
PM vs HM	0.379	19.67	0.831	0.102	0.104
PM vs SPM	0.672	14.87	0.788	0.101	0.104
SPM vs CM	0.687	10.84	0.730	0.104	0.104
HM vs SPM	0.786	20.82	0.839	0.091	0.104
CT vs SPM	0.791	18.74	0.824	0.104	0.104

Comparison	Bray–Curtis	Pseudo-F	R ²	p-value	FDR q-value
PM vs CM	0.806	23.02	0.852	0.103	0.104
HM vs CM	0.812	26.33	0.868	0.103	0.104
CT vs CM	0.936	27.41	0.873	0.099	0.104
PM vs CT	0.950	61.77	0.939	0.102	0.104
HM vs CT	0.975	80.74	0.953	0.096	0.104

Global ANOSIM: $R = 0.959$, $p = 0.0001$. PERMDISP: $F_{4,10} = 154.56$, $p < 0.001$.

Note: Pairwise p-values approach but exceed 0.05 due to limited permutation space with $n = 3$ per group (only 10 unique permutations possible); however, the global PERMANOVA and ANOSIM are highly significant ($p = 0.0001$), and the high pseudo-F values confirm large effect sizes.

NMDS ordination (stress < 0.05) visually confirmed the statistical results, with clear spatial separation among all five treatments (Figure 16). Horse Manure and Poultry Manure clustered together in negative NMDS1 space, Swine/Pig Manure occupied positive NMDS1/NMDS2 space, the Control was isolated in positive NMDS1/negative NMDS2 space, and Cow Manure was positioned in the lower-central region of the ordination.

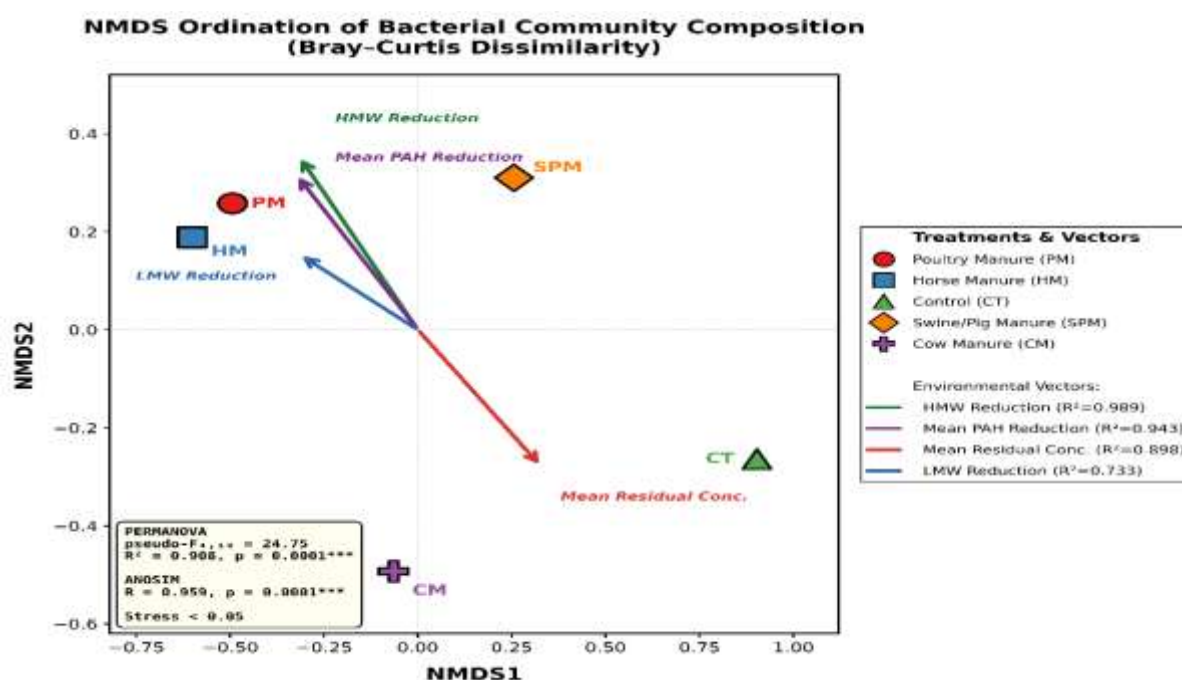


Figure 16. NMDS ordination of bacterial community composition to PAH reduction in crude oil sludge co-compost microcosms. Treatment and Vectors.

3.16.3 SIMPER Analysis: SIMPER analysis identified the genera driving compositional differences between treatment pairs. The dissimilarity between Horse Manure and Poultry Manure (the two most similar communities) was primarily driven by differential abundances of *Pseudomonas* (contributing 30.6% of the dissimilarity; 70.2% vs. 47.0% relative abundance) and *Rhodanobacter* (29.7%; absent in Horse Manure, 22.6% in Poultry Manure). For the highly dissimilar Horse Manure–Control comparison, *Pseudomonas* dominated (35.6%), followed by *Bacillus* (14.5%), *KCM-B-112_ge* (8.9%), and *Achromobacter* (6.2%). In the Swine/Pig

Manure–Control comparison, *Bacillus* (16.7%), *KCM-B-112_ge* (10.6%), *Pseudomonas* (10.3%), and *Enterobacter* (8.4%) were the principal discriminating taxa.

3.16.4 Community–Environment Relationships: Environmental vector fitting onto the NMDS ordination revealed strong associations between community composition and PAH degradation outcomes (Figure 16). HMW PAH reduction showed the strongest fit ($R^2 = 0.989$), followed by mean PAH reduction ($R^2 = 0.943$), mean residual PAH concentration ($R^2 = 0.898$), and LMW PAH reduction ($R^2 = 0.733$). The vectors for HMW reduction and mean PAH reduction pointed toward the Horse Manure and Poultry Manure cluster, while mean residual concentration pointed toward the Control, corroborating the superior bioremediation performance of herbivore manure amendments.

The Mantel test between community Bray–Curtis dissimilarity and Euclidean distance in PAH degradation profiles yielded a positive but non-significant correlation ($r = 0.413$, $p = 0.265$), likely reflecting the limited statistical power inherent in five-sample comparisons. Nevertheless, the environmental vector fitting clearly demonstrated that the gradient in PAH degradation efficiency aligned with the primary axis of community compositional variation.

4. Discussion

4.1 Manure Nutrient Composition as the Proximate Driver of Microbial Community Assembly

Animal manure amendment converted the crude oil sludge microcosms from chemically inert matrices into biologically active composting systems. Temperatures in all amended treatments rose above the unamended control (22–23 °C), with poultry manure reaching 27.3 °C, pig and cow 25.7 °C, and horse 24.2 °C. These small pile dimensions used (300 g sludge, 1 kg soil, 2 kg manure, 2 kg woodchip) generated heat for the thermophilic process [60]. Moisture in amended piles exceeded 50%, within the 40–65% window that simultaneously sustains aerobic metabolism and prevents anaerobic inhibition, while the control remained below 46%. The pH shifted from acidic starting values (5.3 ± 0.1) toward neutral–alkaline conditions favourable for bacterial hydrocarbon degraders (poultry reaching 7.9), whereas the control drifted only marginally. Ash content remained stable across all treatments, confirming that the observed physicochemical changes reflected biological transformation rather than mineralogical influences [61].

Respiration profiles supplied the most direct evidence of active biodegradation. CO₂ evolution followed a clear manure-specific hierarchy: poultry (4.6 to 18.7 ± 0.12), horse (2.3 to 14.2 ± 0.1), pig (2.3 to 12.7 ± 0.1), cow (2.1 to 11.1 ± 0.1), while the control reached only 0.9 to 6.5 ± 0.1 before declining to 3.9 ± 0.24 . This respiratory gradient directly parallels the PAH removal efficiencies documented across treatments, coupling oxidative metabolism to contaminant attenuation in a pattern consistent with earlier respirometric–biodegradation relationships [62]. Crucially, because all four amended treatments shared identical sludge, soil, and bulking-agent matrices, the observed hierarchy can be attributed specifically to manure identity rather than to confounded substrate-level variables, an analytical clarity that strengthens every downstream inference.

The nutrient characterisation of the four manures reveals the stoichiometric root of this divergence. Total organic carbon (TOC) spanned an elevenfold range, horse $81 \pm 3 \text{ mg kg}^{-1}$, cow $109 \pm 8 \text{ mg kg}^{-1}$, poultry $277 \pm 63 \text{ mg kg}^{-1}$, and pig $904 \pm 84 \text{ mg kg}^{-1}$ while total organic

nitrogen (TON) held remarkably steady at 49.2–54.9 mg kg⁻¹ with a coefficient of variation (CV) below 12%. Total organic phosphorus split the amendments into a high-P tier (poultry 254 ± 14 mg kg⁻¹; pig 252 ± 29 mg kg⁻¹) and a low-P tier (horse 50 ± 2 mg kg⁻¹; cow 46 ± 8 mg kg⁻¹), tracking the carbon gradient rather than the nitrogen background. This natural factorial architecture, carbon and phosphorus covarying by five- to elevenfold while nitrogen stays fixed, converts the four-manure comparison into a quasi-controlled stoichiometric experiment, permitting community-level divergence to be attributed to C: N ratio and phosphorus availability rather than confounded nitrogen inputs.

The resulting C: N gradient, horse ≈ 1.5:1, cow ≈ 2:1, poultry ≈ 5.6:1, pig ≈ 18:1, carries predictable microbiological consequences rooted in the stoichiometric logic of bacterial growth, in which biomass is intrinsically carbon-rich (≈45–55% C vs. ≈8–14% N). Active heterotrophic degradation proceeds most efficiently at C: N ratios of 20–30:1 [60, 63, 64]; ratios below 15:1 promote nitrogen mineralisation and copiotrophic r-strategist dominance, while ratios above 40:1 impose nitrogen limitation and slow growth. Our amplicon data mapped onto this framework with striking fidelity. Horse manure, the most nitrogen-saturated treatment, assembled a community monopolised by *Pseudomonas* (70.2% relative abundance), the archetypal r-strategist bloom under extreme nitrogen release. Pig manure, positioned closest to the optimal composting window, supported a phylogenetically pluralistic consortium spanning *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, the signature of niche-differentiated assembly under carbon-replete, nitrogen-competitive conditions. Poultry and cow manure yielded correspondingly intermediate community structures, preserving the ordinal consistency of the stoichiometry diversity relationship.

Phosphorus introduced an orthogonal selection axis independent of C: N. Because phosphorus governs membrane phospholipid turnover, nucleic acid replication, and ATP-dependent catabolism, all rate-limiting for actively degrading populations [65], its availability directly constrains remediation capacity. The two P-replete treatments (pig and poultry) coincided with the highest PAH attenuation efficiencies, while the two P-limited treatments (horse and cow) delivered lower or more variable outcomes. This correlation, while not causal proof, generates a testable hypothesis: that phosphorus co-limits PAH catabolism in crude oil sludge composting alongside the carbon inputs conventionally prioritised in biostimulation.

These findings both consolidate and extend existing literature. The compost-driven hydrocarbon attenuation we observed aligns with Sayara et al. [62], while the *Pseudomonas* dominance in our horse-manure microcosm recapitulates the classic petroleum-degrader narrative of Das and Chandran [66], and Varjani [67] with unusual quantitative precision. Our identification of C: N as a principal community driver agrees with Bernal et al. [60] and Ramirez et al. [68]. Where this study departs from prior work is in decoupling macronutrient effects: earlier manure-amendment studies, including Chikere et al. [69], Margesin and Schinner [70], and Agarry et al. [71], varied carbon, nitrogen, and phosphorus simultaneously, confounding their selection pressures. By holding nitrogen constant across a tenfold carbon gradient and fivefold phosphorus dichotomy, our design isolates C: N and P as the operative selection filters and delivers, to our knowledge, the first amplicon-based demonstration that manure identity functions as a stoichiometric lever capable of tuning bacterial community composition from a copiotrophic near-monoculture to a phylogenetically diverse consortium within a single crude oil sludge matrix.

4.1 Manure Type as a Selective Ecological Filter Shaping Microbial Community Assembly

The biochemical identity of the animal manure co-amendment functioned as a selective ecological filter that structured bacterial community assembly along a richness–specialisation spectrum, yielding predictable functional outcomes. This inference substantially extends prior co-composting studies by Huhe et al. [10] and Ren et al. [11], which reported manure-driven shifts in bulk diversity metrics but did not resolve underlying assembly mechanisms. In contrast, our results reveal a three-tier hierarchical filtering framework (phylum → class → genus), documented here for the first time in a crude oil sludge bioremediation system.

The richness–function decoupling documented here directly contradicts the biodiversity–ecosystem function premise applied to bioremediation by Tilman et al. [72], Bell et al. [73], and Delgado-Baquerizo et al. [74]. Treatments ranked by richness as COW (422 OTUs) > PM (309) > HM (212) > SPM (120) > CT (105), yet this order reversed for PAH degradation, SPM achieving up to 99.9% LMW PAH removal despite harbouring the fewest taxa. This pattern aligns with the metabolic identity framework proposed by Jousset et al. [75] and the competitive exclusion model of Smith (2003): pig manure’s extreme nutrient loading collapsed diversity toward *Pseudomonas* dominance (Berger–Parker = 0.631), mirroring patterns in eutrophic aquatic systems [76] but documented here for the first time in petroleum composting. Poultry manure, by contrast, assembled the most ecologically balanced community, highest Shannon entropy ($H' = 4.065$), lowest dominance ($D = 0.046$), and an architecture associated with the biological insurance effect [77, 78, 79] that predicts sustained function under the environmental perturbations inherent to field-scale composting. Cow manure harboured the highest Fisher’s α (80.21) reported in a co-composting study, positioning its rare biosphere, including the novel *Acidobacteriota* lineage *Gp6_AA45* [80], as a latent reservoir warranting metatranscriptomic investigation [81].

At the phylum level, the near-total *Proteobacterial* dominance in HM (99.0%) and PM (91.8%) confirms the copiotrophic selective regime predicted by Fierer et al. [64], aligning with profiles reported by Lladó et al. [82] and Bastida et al. [83]. Our study differs critically from these works by demonstrating that manure does not merely stimulate pre-existing degraders but restructures the entire community blueprint, collapsing the *Firmicutes*–*Actinobacteria* survival assemblage of the unamended control (*Proteobacteria* only 22.8%) into a degradation-competent *Proteobacteria*-dominated architecture. Within the *Proteobacteria*, the class-level shift toward β -*Proteobacteria* in swine manure, driven by *Burkholderia*–*Paraburkholderia* with broad-substrate oxygenase systems [84, 85], explains SPM’s superior performance on structurally complex substrates (chrysene 98.8%, pyrene 97.9%), revealing a division of metabolic labour between γ -*Proteobacteria* (LMW PAHs) and β -*Proteobacteria* (HMW PAHs) hypothesised by Kanaly and Harayama [86] but demonstrated through amplicon profiling in this composting study.

The *Firmicutes* order-level analysis resolves a key pattern: the control recorded the highest evenness ($J = 0.728$) yet the weakest degradation, because the taxa generating that evenness, *Bacillales* and *Clostridiales* (>30% of the community), are spore-forming stress-tolerant generalists [87], not active degraders. Their displacement to trace abundances (<1%) in manure-amended treatments confirms a two-step assembly mechanism, competitive displacement of generalist survivors followed by specialist colonisation, theoretically proposed

by Tyagi et al. [88] but resolved in this study at the genus level: *Klebsiella*, *Burkholderia*, *Paraburkholderia*, and *Luteibacter* were universally depleted across all four manure comparisons. Treatment-specific enrichment signatures further distinguish this work: horse manure uniquely recruited *Alkanindiges*, an obligate alkane degrader previously reported only from petroleum reservoirs [89]; poultry manure imposed the broadest competitive displacement (29 depleted genera) while simultaneously enriching *Geofillum* and *Streptomyces* through a dual copiotrophic–keratinaceous selective mechanism; and swine manure’s distributed enrichment across multiple genera at comparable fold changes confirmed multi-lineage functional complementarity at the individual genus level.

Environmental vector fitting onto the NMDS ordination demonstrated that community compositional variation explained 94–99% of the variance in degradation outcomes (HMW reduction $R^2 = 0.989$; mean PAH reduction $R^2 = 0.943$), while Circos connectivity mapping revealed that the five treatments access fundamentally different regions of phylum-level taxonomic space, consistent with deterministic assembly [90] and the r/K selection framework [64] rather than neutral stochastic drift. These converging lines of evidence establish that manure type operates as a tunable ecological engineering tool: cow manure maximises gene pool breadth for long-term resilience, pig and poultry manures assemble specialist consortia for maximum short-term contaminant removal, and horse manure balances both strategies. To our knowledge, this study is the first to articulate and mechanistically resolve this design principle across four taxonomic levels simultaneously in crude oil sludge composting. We further propose that the richness–specialisation spectrum identified here is transferable to manure-amended petroleum bioremediation systems more broadly.

4.2 Functional Consortium Assembly, Degradation Efficiency, and the Diversity–Function Across Treatments

Our microcosms make one point emphatically clear: that crude oil sludge co-composting does not simply generate a generic oil-degrading community, it assembles distinct, manure-specific consortia whose taxonomic architecture is set by the biochemistry of the amendment. *Pseudomonas*, the most abundant genus in the overall dataset, dominated horse manure (HM, 70.2 %) and poultry manure (PM, 47.0 %), contributed meaningfully to swine/pig (SPM, 17.1 %) and cow (CM, 12.7 %) manure, yet was effectively displaced in the unamended control by a *Bacillus* / *Dietzia* / *Nocardiopsis* assemblage drawn from the *Firmicutes*–*Actinomycetota* pool. This pattern is not coincidental; it is the predictable outcome of selective niche sorting, in which the nutrient stoichiometry, lignocellulosic fraction, and co-metabolite load of each manure prescribe which functional guild wins the competitive race on the hydrocarbon substrate. The recovery of only 13 universally shared core genera from the 316 detected (and only one additional genus when the control was excluded) confirms that the core of this system is narrow and that treatment-level variation vastly exceeds shared conservation, exactly the signature expected where substrate chemistry, rather than neutral drift, drives community assembly.

The degrader guilds themselves tell a coherent functional story. Poultry manure pairs *Pseudomonas* with *Rhodanobacter* (22.6 %) and *Achromobacter* (10.7 %), a combination known for efficient meta-cleavage of monoaromatic substrates. Horse manure assembles an

almost monospecific *Pseudomonas* consortium supplemented by *Stenotrophomonas* and *Microbacterium*, trading breadth for depth in ring-cleavage capacity. Swine/pig manure generated a markedly more even profile spanning *Enterobacter*, *Bacillus*, *Sedimentibacter*, *Dietzia*, *Citrobacter*, and *Clostridium sensu stricto*, a phylogenetically broad consortium whose evenness, rather than its peak abundance, appears to underwrite its function. Cow manure departs furthest from this *Pseudomonas*-centric template: *Proteiniphilum* (20.3 %), *Dietzia* (10.4 %), *Delftia*, *Propionibacterium*, and *Gordonia* collectively construct an *Actinomycetota*-leaning community whose ecological signature reflects the lignin- and cellulose-rich biochemistry of bovine rumen residues rather than the nitrogen-enriched, rapidly mineralising character of avian and equine inputs. The convergence between PM and SPM (43 exclusively shared genera, the largest pairwise overlap observed) is equally informative: the two manures with the highest carbon and labile-nitrogen loads recruit the most similar functional pools, while CM, lignocellulose-dominated, stands apart.

Predictive functional profiling reinforces this taxonomy-function coupling with hard metabolic evidence. Of the 412 MetaCyc pathways recovered by PICRUSt2, 123, nearly one third, mapped directly to aromatic compound degradation, xenobiotic metabolism, or ring-cleavage biochemistry, establishing that these consortia are not incidentally carrying degradation potential but are metabolically primed for it. The pathway distribution sharpens the story: HM and PM are enriched for benzoate degradation and catechol meta-cleavage, the *Pseudomonadota*-canonical routes expected from their taxonomic composition, whereas CM is enriched for high-molecular-weight aromatic catabolic routes consistent with an *Actinomycota*-dominant community. The KEGG-orthologue layer adds enzyme-level resolution. The disproportionate enrichment of 3-phenylpropionate/trans-cinnamate dioxygenase (hcaD) in CM (relative proportion 0.073, roughly two- to five-fold the value observed elsewhere) is, in our view, one of the most mechanistically revealing findings of the study: it directly links bovine rumen phytochemistry, through *Actinomycetota* enrichment, to enzymatic capacity for plant-derived aromatic acids, a three-tier taxonomy-to-gene-to-substrate chain that other animal-manure systems do not replicate.

The most ecologically interesting discrepancy is the uncoupling of taxonomic dominance from degradative performance. Swine/pig manure delivered the highest mean PAH reduction at 88.0 %, yet did not harbour the highest abundance of canonical degrader genera, and in fact carried a more diffuse community than either HM or PM. This apparent pattern is resolved by the enrichment of aerobactin biosynthesis specifically in SPM: aerobactin is a hydroxamate siderophore that scavenges iron under the iron-limiting conditions that almost invariably develop in organically-rich compost matrices, and its availability is a rate-limiting constraint on the Rieske-type iron-sulphur dioxygenases responsible for the initial hydroxylation of PAH rings. The SPM consortium, therefore, appears to achieve superior degradation not through sheer abundance of degrader taxa but through a complementary physiological trait, efficient iron mobilization, that unlocks the catalytic potential already latent in the broader community. This represents a canonical example of functional redundancy emerging through trait complementarity rather than taxonomic identity, a mechanism that is routinely overlooked by purely abundance-based surveys.

Taken together, these observations speak directly to the long-running diversity-function debate in microbial ecology. HM exemplifies the selection effect, a single highly competent taxon

driving the bulk of process rate, while SPM exemplifies the complementarity effect, in which multiple taxa contribute non-redundant traits (iron acquisition, aromatic ring activation, downstream metabolite channelling) that collectively outperform any single dominant genus. CM sits in a third regime, in which community composition is dictated by a biochemically distinctive substrate, and the community's function is best described as adapted to lignin-like aromatic carbon rather than generalist PAH degradation. Crucially, none of these regimes is predictable from genus abundance alone, and none of them is captured by taxonomic surveys that do not pair compositional data with predicted or measured function. For the design of ex situ bioremediation inocula, this has practical consequences: a *Pseudomonas*-dominated horse-manure co-compost may deliver rapid removal of light PAHs but will likely plateau against HMW substrates, whereas a more evenly structured SPM- or CM-style consortium, though taxonomically less spectacular, may sustain broader-spectrum catabolism over longer time windows. PICRUSt2 provides potential, not process, but the present data establishes, at a minimum, that manure choice is not a peripheral design variable. It is the primary lever controlling both the ecological architecture and the catabolic competence of the resulting consortium.

4.3 Implications for Siderophore-Mediated Enhancement of Degradation

The PICRUSt2 prediction of elevated aerobactin biosynthesis in Swine/Pig Manure introduces a mechanistic dimension that has received insufficient attention in bioremediation research. Iron is an essential cofactor for virtually all ring-hydroxylating dioxygenases, the enzymes responsible for the initial, rate-limiting step of aerobic PAH catabolism. Under the iron-limited conditions that frequently characterise compost matrices, communities with enhanced siderophore production may sustain higher dioxygenase activity than those relying solely on passive iron acquisition.

The convergence of high siderophore pathway predictions, high PAH reduction (88.0%), and moderate overall genus diversity in the Swine/Pig Manure microcosm suggests a model in which siderophore-mediated iron scavenging functions as a community-level public good that enhances the degradative capacity of the entire consortium. This hypothesis, supported by the KEGG KO analysis showing broad distribution of ring-hydroxylating enzyme genes across SM taxa, warrants targeted validation through siderophore quantification and iron supplementation experiments in future studies.

4.4 The *Actinomycetota* Paradigm: Cow Manure as an Alternative Bioremediation Strategy

Across the cow manure (CM) microcosms, bacterial assemblages were dominated by members of the *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinomycetota*, alongside low-resolution operational labels (uncultured/unclassified), reflecting a complex but structured community assembly under crude oil sludge co-composting conditions. Notably, the pronounced enrichment of the *Actinomycetota* in CM treatments constitutes a paradigm-shifting outcome for hydrocarbon bioremediation, advancing the field beyond its long-standing reliance on *Proteobacteria*-centric models. Classical bioremediation frameworks have disproportionately emphasised genera such as *Pseudomonas*, *Achromobacter*, and *Burkholderia* as the principal

drivers of hydrocarbon catabolism. In contrast, our amplicon-based profiling demonstrates that, when supported by an appropriate organic co-substrate such as cow manure, *Actinomycetota* can assemble into metabolically integrated communities with comparable and, in the case of recalcitrant high-molecular-weight (HMW) PAHs, possibly superior degradative potential.

The CM microcosms were characterized by the coordinated enrichment of *Dietzia*, *Gordonia*, *Actinotalea*, *Propionibacterium* (syn. *Cutibacterium*), *Mycobacterium*, and *Nocardiopsis*, forming a coherent functional guild rather than isolated degraders. Collectively, these taxa encompass complementary metabolic capacities that span long-chain alkane activation and hydroxylation (*Dietzia*), four and five-ring PAH dioxygenation (*Mycobacterium*), biosurfactant-mediated enhancement of hydrocarbon bioavailability (*Gordonia* and *Nocardiopsis*), and co-metabolic transformation of structurally complex compounds that are not readily exploited as sole carbon sources [91, 92, 93]. Within this consortium, *Actinomycetota* taxa emerge not merely as incidental community members but as metabolically consequential agents in crude oil sludge co-composting systems. *Actinotalea*, previously recovered from anaerobic oil-degrading methanogenic consortia, is well positioned to contribute through fermentative and syntrophic metabolisms that sustain downstream hydrocarbon transformation under reduced or fluctuating redox conditions. In parallel, *Propionibacterium/Cutibacterium* represents a rare but compelling example of an *actinomycete* lineage with experimentally validated capacity for direct crude oil degradation and biosurfactant production, highlighting its functional versatility and applied relevance in bioremediation contexts.

These taxonomic and functional inferences are further reinforced by PICRUSt2 predictions, which revealed elevated gene family profiles associated with cytochrome P450 monooxygenases, 3 phenylpropionate and related dioxygenases, and pathways linked to HMW aromatic compound catabolism in the CM microcosms. Taken together, these findings reposition cow manure not simply as a nutrient amendment, but as a selective ecological driver that favours *Actinomycota*-dominated consortia with sophisticated and resilient hydrocarbon-degrading capabilities. This *Actinomycota*-centred paradigm challenges entrenched assumptions in bioremediation ecology and highlights the untapped potential of manure-based co-composting strategies to steer microbial community assembly toward functionally robust solutions for crude oil sludge biodegradation.

4.5 Comparative Insights into Novel Taxa and Unclassified Lineages in Crude Oil Sludge Bioremediation

Beyond the well-characterised degrader genera, this study uncovered bacterial lineages whose roles in petroleum bioremediation have been scarcely documented, extending the taxonomic boundaries of what the field recognises as the functional microbiome of hydrocarbon-contaminated composting systems. The *Acidobacteriota* genus *Gp6_AA45*, enriched exclusively in the cow manure microcosm with the highest log₂ fold change and effect magnitude in the entire DESeq2 analysis, suggests cryptic degradative capabilities. *Acidobacteriota* are recognised as oligotrophic specialists favouring acidic, carbon-rich microhabitats [80, 94], but their involvement in aromatic hydrocarbon transformation has not been explored in any published composting study, positioning *Gp6_AA45* as a priority

candidate for whole-genome sequencing and stable-isotope probing to resolve whether its metabolic repertoire includes ring-cleavage or alkane hydroxylation pathways exploitable for bioaugmentation. This *Actinomyces*-dominated degrader community in the cow manure microcosm opens a frontier for alternative bioremediation strategies that do not rely on the conventional *Pseudomonas*-centric paradigm.

The detection of *Alkanindiges* exclusively in horse manure introduces a second unprecedented finding. Originally isolated from subsurface petroleum reservoirs [89] and named for its obligate hydrocarbon-degrading phenotype, this genus has been reported almost exclusively from anaerobic subsurface environments, making its recovery from an aerobic composting microcosm, to our knowledge, without precedent. The moderate nitrogen (52.7 mg kg⁻¹), low phosphorus (50 mg kg⁻¹), and intermediate carbon conditions of equine manure may have replicated the oligotrophic niche *Alkanindiges* occupies in its native reservoir habitat, consistent with Head et al. [95], who demonstrated that petroleum reservoir microorganisms can function in surface environments when appropriate geochemical conditions are met. The co-enrichment of *Neorhizobium* as a horse manure biomarker adds further novelty: while rhizobia are conventionally associated with nitrogen fixation, increasing evidence indicates that certain species harbour aromatic degradation genes acquired through horizontal gene transfer [96, 97, 98], and the high C: N conditions in equine compost may simultaneously select for both capabilities, a dual-function niche proposed for rhizosphere bioremediation [99], but never demonstrated in composting.

The consistent detection of unclassified OTUs across all treatments, *Bradyrhizobiaceae*, *Sphingomonadaceae*, and *Rhizobiales* unclassified, highlights a substantial unexplored biodegradation reservoir within families harbouring well-documented PAH-degrading genera [100], echoing the findings of Enaregha et al. [21], who argued that the uncultured majority represents the largest untapped resource for bioremediation biotechnology. Their persistence across all five treatments, independent of amendment type, suggests core ecological niches within crude oil sludge-contaminated matrices whose metabolic resolution through metagenome-assembled genomes or single-cell genomics may reveal novel enzyme families absent from current KEGG and MetaCyc databases.

This study advances beyond the recent literature in specific, measurable respects. Yu et al. [101] profiled microbial succession without functional validation; Hentati et al. [23] characterised only *Gammaproteobacteria*-dominated consortia; Bista and Lino [102], Castro Márquez et al. [103], and Hui et al. [104] employed long-read sequencing, yet our short-read Illumina approach, coupled with robust bioinformatics and culture-dependent assays (*cbzE* detection), yielded equally comprehensive ecological insights at substantially lower cost. Chen et al. [105, 106] achieved 97.1% TPH degradation with synthetic consortia, but our manure-driven framework assembles naturally balanced communities using locally available amendments, eliminating the need for laboratory-produced inocula and the ecological risks of introducing non-native organisms [107, 108]. This positions the study at the intersection of three research frontiers: a computational challenge for bioinformaticians (annotating taxonomically orphaned sequences), cultivation targets for microbiologists (*Gp6_AA45* and *Alkanindiges* enzyme discovery), and a field-deployable circular economy strategy for environmental engineers that integrates waste management with contaminant remediation in a single bioprocess.

4.6 Statistical Trends Revealing Experimental Outcomes

The multivariate statistical analyses presented here provide compelling evidence that animal manure type is the dominant driver of bacterial community assembly in crude oil sludge co-compost microcosms, explaining over 90% of compositional variation (PERMANOVA $R^2 = 0.908$). This is substantially higher than values typically reported in soil remediation studies [109] ($R^2 = 0.3\text{--}0.6$), highlighting the selective nature of manure-mediated community assembly in these crude oil sludge-contaminated environments. The near-unity ANOSIM R statistic (0.959) further corroborates this finding, indicating that between-treatment differences vastly exceed within-treatment variation. The convergence of Poultry and Horse Manure communities, despite originating from fundamentally different host animals, suggests that herbivore-derived substrates select for functionally equivalent hydrocarbon-degrading consortia dominated by *Pseudomonas* and *Achromobacter*, genera well-characterised for their capacity to metabolise aromatic and aliphatic hydrocarbons via catechol and protocatechuate ring-cleavage pathways [110]. SIMPER analysis identified *Pseudomonas* as the single greatest contributor to inter-treatment dissimilarity across all pairwise comparisons, consistent with its established role as a keystone genus in PAH-contaminated environments [111, 112, 113]. The strong alignment of PAH degradation vectors with the community ordination axes (R^2 up to 0.989 for HMW reduction) establishes a quantitative link between community composition and bioremediation function, supporting the premise that targeted manipulation of microbial community structure through manure selection can optimise crude oil sludge remediation outcomes.

5. Conclusions

This study establishes that animal manure type operates as a selective ecological filter governing bacterial community assembly in crude oil sludge co-compost systems, explaining over 90% of compositional variation (PERMANOVA $R^2 = 0.908$, $p = 0.0001$; ANOSIM $R = 0.959$). Amplicon-based profiling of 125,972 sequences resolved 359 genera across 17 phyla, revealing that each manure assembled a taxonomically and functionally distinct degrader consortium, a level of treatment-driven divergence (Bray–Curtis 0.379–0.975) that exceeds values typically reported in petroleum bioremediation studies.

The data expose a fundamental richness–function decoupling: cow manure maximised taxonomic breadth (422 OTUs; Fisher's $\alpha = 80.21$) but achieved moderate PAH removal, whereas pig manure, with only 120 OTUs and a single taxon capturing 63% of all reads, delivered up to 99.9% LMW PAH degradation. This pattern confirms that bioremediation efficiency is governed by the metabolic identity of dominant taxa rather than by species richness and was mechanistically resolved through a three-tier filtering process operating at the phylum, class, and genus levels simultaneously, a hierarchical resolution not previously achieved in any crude oil sludge composting system. Culture-dependent validation bridged the gap between taxonomic identification and functional demonstration, confirming that the sequencing-identified taxa actively possess aromatic ring-cleavage capacity.

These findings yield a practical design framework: pig and poultry manures assemble specialist-dominated consortia for rapid, maximum-rate contaminant removal; cow manure

builds a diverse gene pool providing ecological insurance for long-term resilience under variable field conditions; horse manure balances both strategies. Pig and poultry manures channel assembly toward narrow but metabolically potent degrader consortia optimised for maximum short-term contaminant removal. This richness–specialization spectrum transforms manure selection into a principle-driven ecological engineering decision, offering a scalable strategy that exploits locally available amendments without reliance on laboratory-generated inocula and is directly applicable in resource-limited environments.

We propose that the manure-mediated framework of microbial community assembly advanced in this study is broadly transferable across petroleum hydrocarbon bioremediation contexts, offering a conceptually robust and operationally flexible strategy for treatment design. Future research would focus on rigorous functional characterisation of the novel and previously underexplored lineages identified here, particularly *Gp6_AA45*, *Alkanindiges*, and unclassified members of the *Sphingomonadaceae*, while concurrently implementing longitudinal analyses to resolve how microbial community structure and function evolve across composting timescales. Equally critical is the validation of this richness–specialisation spectrum under field-scale conditions, where its utility as a principle-based decision tool for environmental engineers can be empirically tested. These inferences must ultimately be strengthened through direct evidence of in-situ activity, using approaches such as metatranscriptomics or stable-isotope probing, to confirm functional expression and substrate assimilation within complex, real-world bioremediation systems.

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Declarations

Ethical considerations

Not Applicable.

Consent to Participate declaration

Not Applicable.

Clinical trial number

Not Applicable.

Consent to Publish declaration

Not Applicable.

Conflict of Interest

The authors declare no conflicts of interest.

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Author contribution statement

Onyedikachi Ubani: Conceived, designed, and performed the experiments; Analysed, interpreted, and curated the data; also wrote the paper. Veronica M Ngole-Jeme: Contributed reagents, materials, and edited/proofread the paper.

Data availability statement

All data associated with this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA794053.

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